Promoters: **Prof. dr. ir. Peter Bossier**
Department of Animal Production, Faculty of Bioscience Engineering, Ghent University.
(peter.bossier@Ugent.be)

Prof. dr. Patrick Sorgeloos
Department of Animal Production, Faculty of Bioscience Engineering, Ghent University.
(patrick.sorgeloos@Ugent.be)

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(nobert.dekimpe@Ugent.be)

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(elsjm.vandamme@Ugent.be)

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Department of Biology, Dalhousie University, Canada.
(tmacrae@dal.ca)

Prof. dr. James S. Clegg
Bodega Marine Laboratory,University of California Davis, USA.
(jsclegg@ucdavis.edu)

Prof. dr. Eric Cox
Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University.
(eric.cox@Ugent.be)

Prof. dr. Frank Pasmans
Department of Pathology, Bacteriology and Poultry Disease, Faculty of Veterinary Medicine, Ghent University.
(frank.pasmans@Ugent.be)

Yeong Yik Sung

Protection of *Artemia* from vibriosis by heat shock and heat shock proteins

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title: Bescherming van *Artemia* tegen vibriose door warmtebehandeling en hitteschokproteïnen

Cover page: *Artemia* nauplii (figure adapted from www.osc.mun.ca/ardf/artemia.html)

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List of abbreviations and units

°C	Degree Celsius
%	Percentage
β-glucans	Beta-glucans
µE/m2s	Micro Einstein per second and square meter
µg	Microgram
µm	Micrometer
µl	Microlitre
λ	Wavelength
g	Relative centrifugal force or G force
±	Approximately
/	Per
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
CCAP	Culture Collection of Algae and Protozoa Department, Scotland
cDNA	Complementary deoxyribonucleic acid
cfu	Colony forming unit
CHO	Chinese hamster ovary
cm	Centimeter
CTR	Control treatment
d	day
Da	Dalton
DAB	Diaminobenzidinetetrahydrochloride dihydrate
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked ImmunoSorbent Assay
exp	Exponential growth phase
FAO	Food and Agricultural Organization of the United Nations
FASW	Filtered and autoclaved seawater
FT	Falcon tube
g	Gram
GART	Gnotobiotic *Artemia* test system
GenBank	Genetic sequence database of the National Institute of Health, USA

GH	Growth hormone
GR	glucocorticoid receptor
g/l	Gram/liter
h	Hour
HS	heat shock
Hsc	Heat shock cognate
HSF	Heat shock factor
Hsps	Heat shock proteins
IGF	Insulin-like growth factor
IL1	Interleukin 1
IL	Individual length
ISSCAAP	International standard statistical classification for aquatic animals and plants
kDa	kilo Dalton
kg	Kilogram
kW	Kilowatt
l	litre
LMG	Laboratory of Microbiology of the Ghent University (Culture Collection)
LPS	Lipopolysaccharides
LRRs	Leucin-rich repeats
LVS…	Strain … isolated by Laurent Verschuere
M	Molar
MA	Marine agar 2216
mg	Milligram
MHC	Major histocompatibility
min	Minute
ml	Milliliter
mm	Millimeter
mM	Milimolar
mnn…	Mutants with deleted genes involved in the extension of
mRNA	Messenger ribonucleic acid
MTT	-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide
n	Number of replicates
ng	Nanogram

NLHS	Non-lethal heat shock
NSS	Nine salt solution
nm	Nanometer
NO	Nitric oxide
OD	Optical density
oPRL	ovine prolactin
p	protein
p	Statistical p-value obtained
PCR	Polymerase chain reaction
pH	Measure of the acidity of a solution
ppt	Part per thousand
proPO	Prophenoloxidase
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
R2	Coefficient of determination
r	Coefficient of correlation
RNA	Ribonucleic acid
rpm	Rotations per minute
RNA	Ribonucleic acid
s	Second
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sHsps	Small heat shock proteins
TBT	Tributyltin
TLR	Toll like receptor
TNF	Tumor necrosis factor
US$	United states currency (Dollar)
USA	United States of America
v/v	Volume per volume
VC	*Vibrio campbellii*
VP	*Vibrio proteolyticus*
w/v	Weight per volume
WT	Wild type

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CHAPTER 1

Introduction

1. INTRODUCTION

1.1. Importance of aquaculture

Capture fisheries exploit much of the world's oceans and pressure on this resource will increase as the human population expands from 6 billion people in 2000 to an estimated 9-10 billion by 2050 (FAO, 2002). The per capita consumption of seafood will increase significantly due to heightened consumer awareness of health and nutritional benefits derived by including fish in the diet, and because seafood is a good source of animal protein available at reasonable price (Carlberg and Van Olst, 2001). The average per capita consumption of seafood reached 18 kg in 2001, almost five times more than in 1961, and it is projected to increase to 20 kg by 2030 (FAO, 2004). To meet this demand, the supply of edible fish and shellfish must grow to at least 180 million tons (Carlberg and Van Olst, 2001).

While total production from capture fisheries had fallen recently, aquaculture, comprised primarily of fish, shrimp and shellfish farming is an important alternative to fulfill increasing demands for food. In 50 years, production has increased from less than a million tonnes per year to 59.4 million tonnes in 2004, with a value of US$70.3 billion (FAO, 2004). Aquaculture has grown at an average rate of 8.8% per year since 1970, compared with only 1.2% for capture fisheries and 2.8% for terrestrial meat production. Aquaculture is one of the fastest growing food production sectors in the world. Indeed, according to FAO statistics, aquaculture provided nearly 50% of the annual world fisheries production of 120 million metric tonnes in 2004 (FAO, 2006). Of this, half is comprised of finfish, a quarter is aquatic plants and the remaining quarter is crustacea such as shrimp, prawns, lobster, crabs and molluscs, including clams, oysters and mussels (Fig. 1.1).

Over 240 different aquatic animals and plants were cultured in 2004. By volume, the cyprinids emerge as the most important cultured species, with 18.2 million tonnes valued at US$16.3 billion. Ostreidae (oysters) are a distant second at 4.6 million tonnes, followed

closely by molluscs at 4.1 million tonnes. The aquatic animals used in aquaculture and their rankings according to quantity and value are listed (Table 1.1).

Table 1.1. Top ten ISSCAAP species groups of aquatic animals used for aquaculture in 2004 (adapted from FAO, 2006)

Species group	Production tonnes	Production % of world total	Value billion US$
Carp and other cyprinids	18 303 847	40.3	16.4
Oysters	4 603 717	10.1	2.8
Clams, cockles, arkshells	4 116 839	9.1	3.3
Miscellaneous freshwater fish	3 739 949	8.3	6.0
Shrimps, prawns	2 476 023	5.5	9.7
Salmon, trout, smelts	1 978 109	4.4	6.6
Mussels	1 860 249	4.1	1.0
Tilapia and other cichlids	1 822 745	4.0	2.2
Scallops, pectens	1 166 756	2.6	1.7
Miscellaneous marine molluscs	1 065 191	2.3	0.6
Other species	4 334 931	9.5	12.9
Total	45 468 356	100.0%	63.4

Nearly 90% of the world's aquaculture production originates in nine Asian countries that produce low value carp and seaweed for domestic consumption, and marine shrimp and molluscs for export (Fig. 1.2). China is the largest producer with more than 41.3 million tonnes or 69.6% of the total yield, and the remaining Asia-Pacific region contributes 21.9% (FAO, 2006). The Western European region adds 3.5% with 2.1 million tonnes while Central and Eastern Europe generate 0.4% or 250 000 tonnes, supplying high value salmon and trout for export, and carp for domestic consumption. Latin America, the Caribbean and North America supply 2.3% and 1.3% respectively, including large amounts of marine shrimp and salmon for export. Yield from Sub-Saharan Africa represents 0.2% whereas the Near East and North Africa account for 0.9%, of total global aquaculture production for 2004.

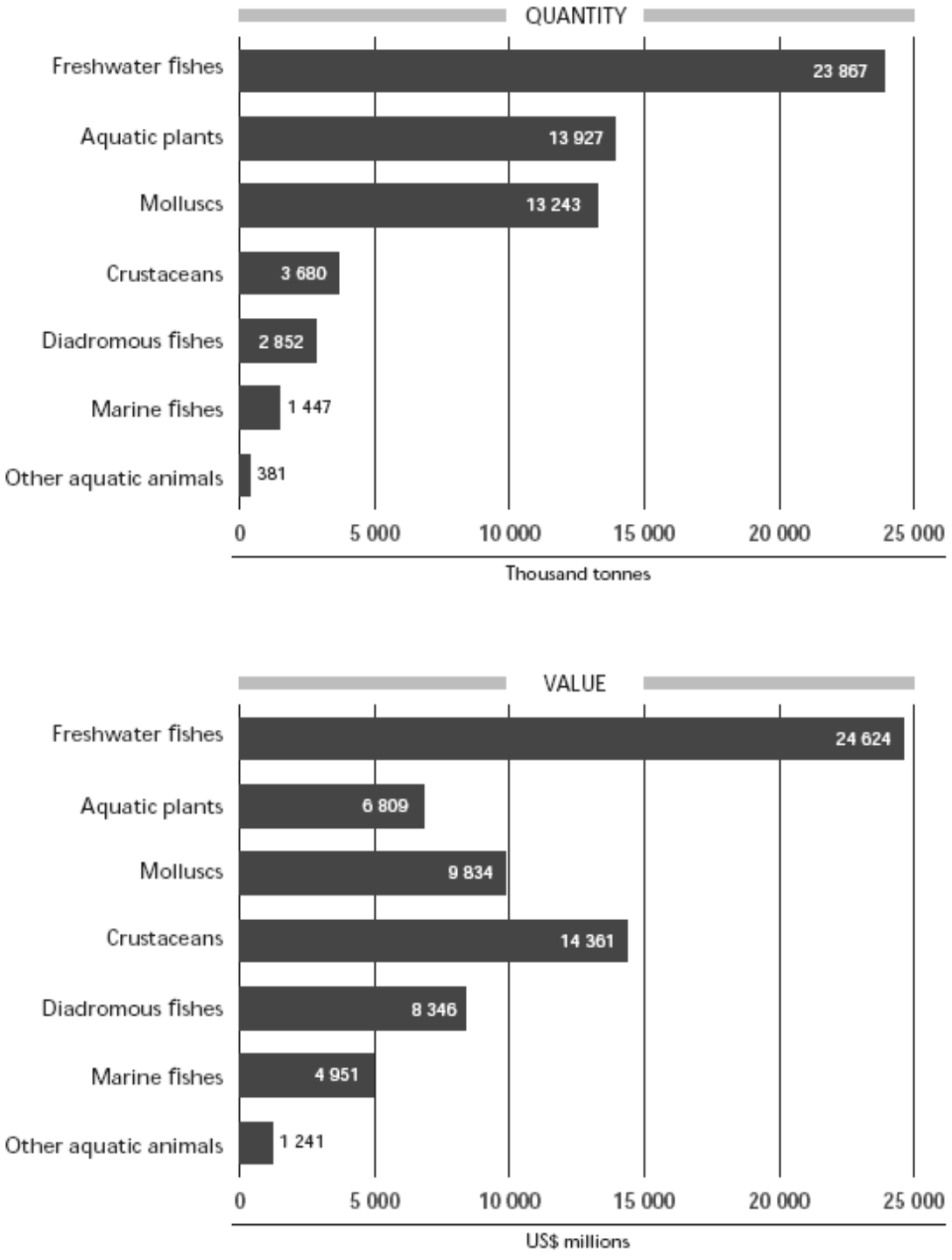

Fig. 1.1. Aquaculture production in 2004 (adapted from FAO, 2006)

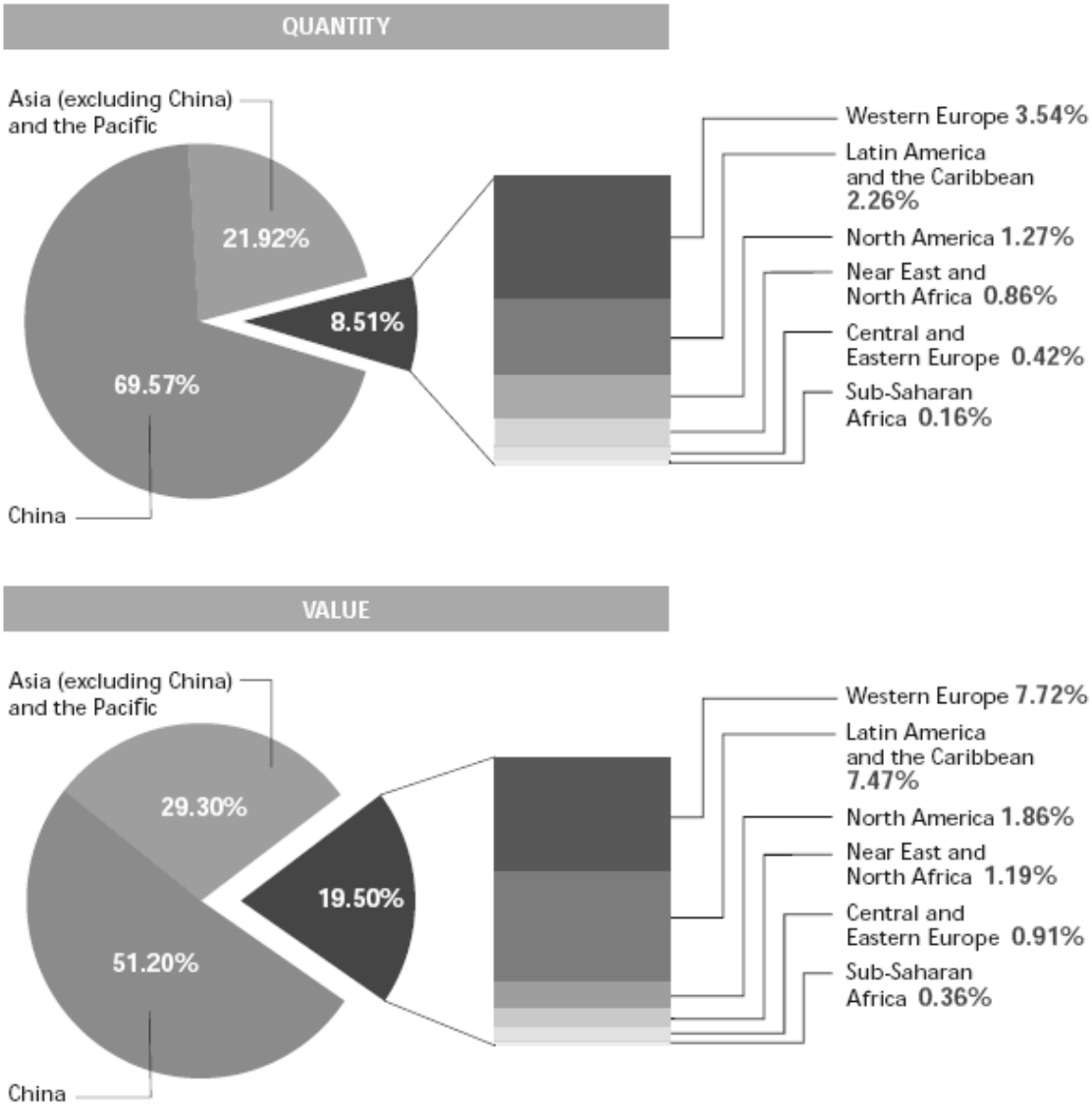

Fig. 1.2. Aquaculture production by region in 2004 (adapted from FAO, 2006)

1.2. Disease – A major constraint for aquaculture

Disease is a major impediment to the development of the aquaculture industry. Disease occurs in all fish and shrimp life stages during culture, resulting from the proliferation of pathogenic and opportunistic microorganisms. Fish and shrimp are affected by many pathogens including protozoa, fungi, and viruses (Lightner and Redman, 1998) (Table 1.2), but bacteria such as *Vibrio* sp. are by far the most serious, causing massive

mortalities of cultured organisms worldwide (Lavilla-Pitogo et al. 1998, Chen et al. 2000; Austin and Zhang, 2006). Disease associated with vibrios, commonly termed vibriosis, is caused by gram-negative bacteria in the family Vibrionaceae. *Vibrio* sp. are included in the chitinoclastic bacterial group associated with shell disease (Cook and Lofton, 1973), and they may enter through wounds and pores in the exoskeleton or via gastro-intestinal routes (Jiravanichpaisal and Miyazaki 1994, Alday-Sanz et al., 2002; Martin et al., 2004). Disease symptoms include septicaemia, partial tail cramping, slow metamorphosis and growth, diffuse abdominal musculature opacity, body malformation and melanisation (Main and Laramore, 1999; Agguire-Guzmán et al., 2004). *Vibrio* sp. are widely distributed in culture facilities and they are opportunistic bacteria that cause disease when the host immune system is suppressed by improper handling, overcrowding, nutritional deficiencies, poor water quality and extreme temperature (Peddie and Wadle, 2005).

Rapid intensification of farming which leads to increased environmental degradation, facilitates vibriosis (Johnson, 1989) and physiological damage caused by parasites may damage fish and shrimp tissues thereby creating an ideal location for *Vibrio* infections. The severity of *Vibrio* infections varies with mortalities exceeding 50% for fish (Reed and Floyd, 1996) and shrimp (Table 1.3). In Asia and South America, vibriosis affects significant mortalities in *L. vannamei* and penaeid shrimp (Austin and Zhang, 2006). In Indonesia, financial losses due to *V. harveyi* infections were as high as US$100 million in 1991 (APEC/FAO/NACA/SEMARNAP, 2000).

Apart from vibriosis, other epizootic disease such as ulcerative syndrome causes US$10 million in several Asian countries. Losses due to viral infections such as white spot disease are estimated at US$400 million and 176 million in China and India, respectively, between 1993 and 1994. Moreover, *Piscirickettsia salmonis* causes an annual loss of US$150 million per year in Chile (Subasinghe and Phillips, 2002).

Table 1.2. Common diseases associated with fish and shrimp culture (modified from Main and Laramore, 1999)

Disease	**Host**	**Pathogen**
Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV)	Fish, Shrimp	Rhabdovirus
Runt-deformity syndrome	Shrimp	IHHNV virus
Taura Syndrome Virus (TSV)	Shrimp	Noda-virus
White Spot Syndrome Virus (WSSV)	Shrimp	Virus
Lymphocystis disease	Fish	Iridovirus
Infectious pancreatic necrosis (IPN)	Fish	Birnavirus
Hespes virus disease	Fish (salmonids)	Hespesvirus salmonis
Yellowhead Virus (YHV)	Shrimp	Virus
Vibriosis	Fish, Shrimp	Bacteria (*Vibrio* sp.)
Bacterial Gill and Kidney disease	Fish	Bacteria
Bacterial Hemorrhagic Septicemia	Fish	Bacteria
Necrotizing hepatopancreatitis	Shrimp	Gram negative bacteria
Mycobacteriosis	Fish, Shrimp	Bacteria (*Mycobacterium* sp.)
Epicommensal fouling disease	Shrimp	Bacteria, protozoans, diatoms or blue-green algae
Black spot disease	Shrimp	Unknown but lesions may indicate TSV
Saprolegniasis	Fish	Fungus (*Saprolegnia sp*)
Branchiomycosis (Gill rot)	Fish	Fungus (*Branchiomyces sanguinis*)
“Ich” or White spot disease	Fish	Protozoan (*Ichthyophthirius multifiliis*)
Costiasis	Fish	Protozoan (*Ichthyobodo necatrix*)

Table 1.3. Mortality caused by luminescent vibrios in different shrimp species (modified from Defoirdt, 2007)

Shrimp species	**Mortality level**	**References**
Kuruma prawn (*Penaeus japonicus*)	Mass mortality	Liu et al., 1996
Ridgeback prawn (*Sicyonia ingentis*)	Up to 55% mortality	Martin et al., 2004
Tiger prawn (*Penaeus monodon*)	Mass mortality	Karunasagar et al., 1994; Lavilla-Pitogo et al., 1990
White shrimp (*Litopenaeus vannamei*)	Up to 85% mortality	Aguirre-Guzman et al., 2001
Brine shrimp (*Artemia franciscana*)	Between 45 to 80% mortality	Soto-Rodriguez et al., 2003

1.3. Alternatives in disease prevention and control

The conventional way to control vibriosis and other types of bacterial disease during aquaculture is by application of antibiotics and pharmacologically active compounds or drugs (Vaseeharan et al., 2004). The use of chemotherapeutic drugs is effective but may have severe negative impacts on living organisms and the environment (Hameed and Balasubramaniam, 2000). For example, inappropriate antibiotic use, especially when pathogens are not evident, leads to development of resistant pathogens (Kautsky et al., 2000; Defoirdt, 2007). Moreover, antibiotics contaminate the environment and may accumulate in tissues of seafood (Vadstein, 1997). In order to solve this problem, several bio-control strategies and environmentally friendly prophylactic alternatives (apart from vaccines which are not discussed here) have been developed.

Green water techniques

The “green water” technique, based on the addition of selected microalgae strains in closed aquaculture systems (Papandroulakis et al., 2001), is perhaps the most commonly used

bio-technique to control disease, demonstrating promising results with aquaculture organisms. *Isochrysis galbana* enhances survival of sea bass larvae (Cahu et al., 1998) and incubation with *Spirulina platensis* enhances the immune system of carp (Pulz and Gross, 2004). Addition of cyanobacteria attenuates disease caused by bacteria and virus by stimulating secretion of an antioxidative substance and immunologically active molecules and virostatic compounds (Cohen, 1999). *Tetraselmis* sp. protects fish and shrimp against the detrimental effects of pathogenic bacteria by producing antibacterial substances (Kellam and Walker, 1989; Austin and Day, 1990; Austin et al., 1992; Salvesen et al., 2000). Together, these studies indicate that micro-algae attenuate disease in diverse ways, contributing to the well-being of cultured animals.

Probiotics

The use of probiotic bacteria as a prophylactic solution to disease is gaining acceptance in aquaculture. Upon addition, probionts improve organismal health, enhance larval survival and prevent the proliferation and colonization of opportunistic and/or pathogenic bacteria in intensive aquaculture rearing systems (Verschuere et al., 2000a; Fanzafar, 2006). Besides contributing to intestinal microbial balance (Talwalkar, 2003, Fanzafar, 2006), probionts (e.g. *Lactobacillus* spp) produce inhibitory compounds and chemical substances such as acids, hydrogen peroxide and bacteriocins that antagonize pathogen growth (Reid, 1999; Vázquez et al., 2005). Probionts also compete for adhesion sites in the gut, reducing colonization by opportunistic bacteria (Strøm and Ringø, 1993). and they may improve water quality (Verscheure et al., 2000b). Moreover they enhance immunity, consequently increasing host resistance to enteric infections (Vaseeharan and Ramasamy, 2003).

Microbially matured water

Microbially matured water is another useful approach to disease control (Verschuere et al., 2000a). Maturation requires pre-treatment of rearing water with a biological filter until non-opportunistic bacteria dominate the resident microbial community. The use of microbially matured water increases the survival and growth of marine fish such as Atlantic halibut (*Hippoglossus hippoglossus*) (Vadstein et al., 1993) and turbot (*Scophthalmus maximus*) (Skjermo and Vadstein, 1999), preventing the growth of opportunistic bacteria.

Immunostimulation

Immunostimulants are biological extracts and synthetic chemicals that stimulate the immune response (Bagni et al., 2005) and which are administered as dietary supplements, by injection or by immersion (Smith et al., 2003). Live bacteria, killed bacteria with attendant bacterions and bacterial antigens, glucans, peptidoglycan and bacterial lipopolysaccharides (LPS) act as potential immunostimulatory compounds in aquaculture (Smith et al., 2003). As one example, β-glucans and polysaccharides stimulate haemocyte exocytosis in shrimp, leading to release of proPO system effectors which eliminate pathogens (Sritunyalucksana and Söderhall, 2000). Moreover, *Aeromonas hydrophila*, either dead or alive, enhances survival of the brine shrimp *Artemia* upon *V. campbellii* challenge. Protection may depend on immune stimulation (Marques et al., 2005) which promotes phagocytic cell function, increases bactericidal activity, and/or stimulates production of non-specific cytotoxic cells (Misra et al., 2004; Skjermo and Bergh, 2004; Bagni et al., 2005; Wang and Chen, 2005).

Heat shock proteins

Hsps mediate the generation of strong innate and adaptive immune responses during many mammalian diseases (Morimoto et al., 1994), leading to formulation of strategies to fight infections. Hsps released into the extracellular environment can elicit cytokine

production. Moreover, Hsps deliver maturation signals and peptides to antigen presenting cells through receptor-mediated interactions (Pockley, 2003), suggesting that Hsps are immunoregulatory agents with potent and widely applicable therapeutic uses. As one example, Hsps are potentially useful for producing cancer vaccines since they bind protein fragments from malignant cells and present them to the immune system, achieving destruction of these cells (Srivastava, 2002b). In this study, the hypothesis that Hsps control disease in aquaculture is explored. The effectiveness of stress-induced endogenous Hsp70 in protecting *Artemia* during pathogenic *Vibrio* challenge is examined. In addition, the feeding of bacterially encapsulated Hsps in advance of an infectious challenge is tested for induction of *Artemia* larvae tolerance against deleterious *Vibrio* effects.

1.5. Thesis outline

The thesis begins with two chapters summarizing related literature (Chapter 2 and 3), four chapters with experimental data showing the effect of heat shock and heat shock proteins on disease resistance in *Artemia* (Chapter 4 to 7), and the final chapter consisting of a general discussion and conclusions (Chapter 8).

In Chapter 2 (Literature overview), Hsps and their diverse roles were summarized. The biological features of the brine shrimp *Artemia* as well as their particular characteristics which enable its use in aquaculture and as a model organism were described.

Chapter 3 (Heat shock proteins in aquatic organisms: Induction, stress tolerance and application) reviews recent evidence on the relationship between stress and Hsp induction, including characterization of abiotic and biotic factors that induce Hsp synthesis in fish and shellfish. The chapter also considers how aquatic organisms profit from increased levels of Hsps. The review concludes with a concise discussion on potential applications of Hsps in aquaculture.

In **Chapter 4 (Non-lethal heat shock protects gnotobiotic *Artemia franciscana* larvae against virulent Vibrios)**, a non-lethal heat shock is tested for its cross-protective effect in *Artemia* exposed to a pathogenic *Vibrio* challenge. Variables investigated include severity of heat shock and recovery time. The relationship between enhanced resistance and expression of endogenous Hsp70 in *Artemia* is described.

Based on the outcome of chapter 4, a complementary study was performed in **Chapter 5 (Exposure of gnotobiotic *Artemia franciscana* larvae to abiotic stress promotes heat shock protein 70 synthesis and enhances resistance to pathogenic *Vibrio campbellii*),** to demonstrate the relationships between Hsp70 and enhanced immunity in *Artemia* larvae. This chapter extends the focus on the differential expression of *Artemia* larvae Hsp70 upon exposure to abiotic stressors such as temperature and osmotic perturbations, and explores the cross-protective effect of these stresses in *Vibrio* challenges. In addition, the effects of these stresses on weight loss, induced thermotolerance, and immune response were evaluated.

Since there is compelling evidence in chapters 4 and 5 that endogenous Hsps protect *Artemia franciscana* against pathogenic vibrios, **Chapter 6 (DnaK, the 70-kDa bacterial heat shock protein, protects *Artemia* larvae from vibrio infection)** examines the protective properties of exogenous Hsps more exhaustively. In this chapter, *E. coli* strains that synthesize different Hsps were constructed and fed to *Artemia* larvae under gnotobiotic conditions. These *Artemia* larvae were then submitted to a *Vibrio* challenge. The results show that ingestion of *Escherichia coli* over-producing prokaryotic Hsps significantly improved larval survival of gnotobiotically cultured *Artemia* upon challenge by pathogenic *V. campbellii*.

The objective of **Chapter 7 (Ingestion of DnaK producing bacteria attenuates *Vibrio* infection of *Artemia franciscana* larvae)** was to better understand how Hsp70 family

members, including DnaK, protect *Artemia* larvae from *Vibrio* infection, work that also illuminates the nutritional properties of bacteria. Several heat stressed bacterial strains were tested with gnotobiotically cultured *Artemia* larvae and their protective effects against *Vibrio* challenge correlated with Hsp70 production.

Chapter 8 (Discussion and Conclusions) recapitulates the results and conclusions presented in Chapter 4 to 7 and considers these findings in relation to the present knowledge and previous findings in relevant fields.

CHAPTER 2

Literature Overview

2. Literature Overview

2.1. The brine shrimp *Artemia*

Artemia characteristics

The brine shrimp *Artemia* is a small branchiopod crustacean found typically in saline lakes, coastal lagoons and salt works worldwide, but they also inhabit brackish and hyper-saline waters because they are highly osmotolerant (Van Stappen, 1996). In their natural environment, *Artemia* survive harsh environmental conditions such as high ultra-violet radiation, extreme temperatures and changing aeration, where other animals would die (MacRae, 2003; Clegg, 2007). Oviparous development in *Artemia* yields cysts comprised of rigid shells covering metabolically inactive dormant embryos that can remain in total stasis for a long time (Criel and MacRae, 2002; Van Stappen, 2002). Upon immersion in seawater under appropriate conditions of temperature and aeration, the cysts hydrate, begin development and rupture within 24 h, releasing free-swimming larvae. The first larval stage, called instar I nauplius, measures about 0.5 mm in length (Van Stappen, 1996). At this stage, nauplii thrive completely on yolk reserves and do not ingest food as the digestive system is only partially functional. After about 8 h, the animal molts into the second larval stage (instar II) and commences feeding on small food particles (Van Stappen, 1996). The larvae then proceed through approximately 15 molts and from the 10th instar stage onward morphological and functional changes such as sex differentiation take place. Adult *Artemia* measure about 1 cm in length and the body thickness of both sexes, including the legs, is approximately 4 mm (Criel and MacRae, 2002).

Artemia are continuous, non-selective particle-filter feeders (Dhont et al., 1993) utilizing algae, bacteria, yeast, detritus and industrially derived food including corn bran, whey powder, rice bran, lactoserum and soybean pellets ranging from 1 to 50 μm in diameter (Dobbeleir et al., 1980). Several factors, including feed quality and quantity, developmental

stage, and culture conditions influence the feeding behaviour of *Artemia* (Marques et al., 2005, Van Stappen, 1996).

The importance of Artemia to aquaculture

Artemia larvae are used widely in the larviculture of fish and shellfish (Sorgeloos et al., 2001). *Artemia* cyst consumption has increased exponentially, in parallel with the development of ornamental industry and aquaculture (Dhont and Sorgeloos, 2002; Lim et al., 2002). More than 1,500 metric tonnes of dry cysts are marketed annually to feed marine and freshwater fish and crustacean species (Dhont and Sorgeloos, 2002). Within the aquaculture industry, shrimp hatcheries are the biggest consumers of *Artemia* cysts, utilizing about 80 to 85% of total market availability, while the remainder is used mainly in marine fish larviculture and the ornamental industry (Dhont and Sorgeloos, 2002).

The ability of *Artemia* to form cysts accounts in part for its convenience as a larval food source (Léger et al., 1986). *Artemia* cysts have a remarkable shelf life. The ease and simplicity of hatching make brine shrimp one of the most convenient, least labor-intensive live foods available for aquaculture. Nutritionally, newly hatched *Artemia* nauplii are high in protein and lipids, constituting an excellent food source for fish and shrimp larvae (Dhont et al., 1993). Furthermore, the ability of *Artemia* to feed on suspended particles allows ingestion of bioencapsulated nutrients, pigments (Sorgeloos et al., 2001), antimicrobial agents (Dixon et al., 1995), vaccines (Campbell et al., 1993), and probionts (Gatesoupe, 1994). Exploitation of these characteristics has led to improvements in larviculture outputs, not only in survival, growth and success of fish and crustacean metamorphosis, but also by reducing the incidence of malformations while improving pigmentation and stress resistance (Van Stappen, 1996; Harzevili, 1998; Ringo and Birkbeck, 1999).

Artemia as test organism

Artemia are used widely for scientific research. With their ability to remain dormant for long periods and resist stress, *Artemia* cysts were used in space radiation experiments (Demets, 1995). The animal is extremely useful for studying the biology of infections or the effect of chemotherapeutic agents on diseases in crustaceans (Overton and Bland, 1981; Criado-Fornelio et al., 1989; Verschuere et al., 1999, 2000b; Marques et al., 2005). They are useful organisms for stress response studies (Clegg et al., 2000a; Frankenberg et al., 2000, MacRae, 2003), feed quality analysis (Marques et al., 2004a, b) and probiont testing (Marques et al., 2005, 2006c). A recently established gnotobiotic *Artemia* test system (GART), where larvae are hatched and grown in axenic conditions, has facilitated research on host-microbe interactions (Marques et al., 2006b). This system has contributed to the elucidation of several biologically-based therapeutic alternatives for potential use in aquaculture (Marques et al., 2006a; Soltanian et al., 2007, Defoirdt et al., 2006a, b). Here, the GART system was employed to examine the potential application of Hsps for disease control in aquaculture.

2.2. Heat shock proteins

The heat shock response was discovered when exposure of the *Drosophila* larval salary gland to elevated temperature was observed to generate puffs in polytene chromosomes (Ritossa, 1962). However, it was not until 1974 that these puffs were proven to represent transcriptional induction of genes encoding heat shock proteins (Hsps) (Tissieres et al., 1974). Hsps occur in all organisms (Lindquist and Craig, 1988). They are constitutively expressed, representing 5-10% of the total protein in healthy growing cells and two or three times that amount when induced by stressors such as heat, cold, nutritional deficiencies, oxygen deprivation and disease (Morimoto, 1998; Pockley, 2003). Consequently, Hsps are

referred to as stress proteins and their up-regulation is generally described as part of the stress response. In eukaryotes, Hsps genes are categorized into several families and named according to their function, sequence homology and molecular mass in Daltons (Da). The families primarily include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and several small Hsps (Table 2.1). Many family members have counterparts, referred to as heat shock cognates (Hscs), that are expressed under normal non-stress conditions.

Induction and regulation of Hsp gene expression

Regulation of heat shock protein gene transcription is mediated by the interaction of heat shock factors (HSFs) with heat shock elements in gene promoter regions (Voellmy, 1994; Morimoto et al., 1994a; Pockley, 2003). Animal and plant HSFs have remarkable structural similarity, but there are significant differences in the complement and activity of family members (Feder and Hofmann, 1999). Some organisms such as yeast (Wiederrecht et al., 1988) and *Drosophila* (Clos et al., 1990) have only one HSF while vertebrates have four (Scharf et al., 1998; Pirkkala et al., 2001). HSF1, HSF2 and HSF 4 are ubiquitously expressed whereas HSF3 appears to be avian specific (Morimoto, 1998). HSF1 is the main transcription factor regulating response to physiological and environmental stress (Morimoto, 1998).

Table 2.1. Major Hsp families (modified from Lindquist et al., 1992)

Protein	Members	Monomer mass (kDa)	Cell localization	Function
Hsp100	ClpA ClpB ClpC Hsp104	80-110	Cytoplasm, nucleolus, nucleus, chloroplast	Thermotolerance, ethanol tolerance, long-term spore viability
Hsp90	Hsp82 Grp94 HtpG	82-96	Cytoplasm, nucleus	Essential for viability; increased concentration required for growth at high temperatures
Hsp70	DnaK, grp78, hsc70, BiP, Kar2, ssa, ssb, ssc, ssd	67-76	Cytoplasm, nucleus, mitochondria, chloroplasts, endoplasmic reticulum	Chaperone required for protein assembly, secretion, protein import into the endoplasmic reticulum and organelles; growth at high temperature
Hsp60	GroEL, Hsp65, cpn60, Rubisco-binding protein	58-65	Mitochondria, chloroplasts	Chaperonin, assembly of oligomeric proteins and folding of monomeric proteins; high concentration required for growth at elevated temperature
sHsps	Many	18-40	Cytoplasm, nucleus	Protection from stress, apoptosis inhibition

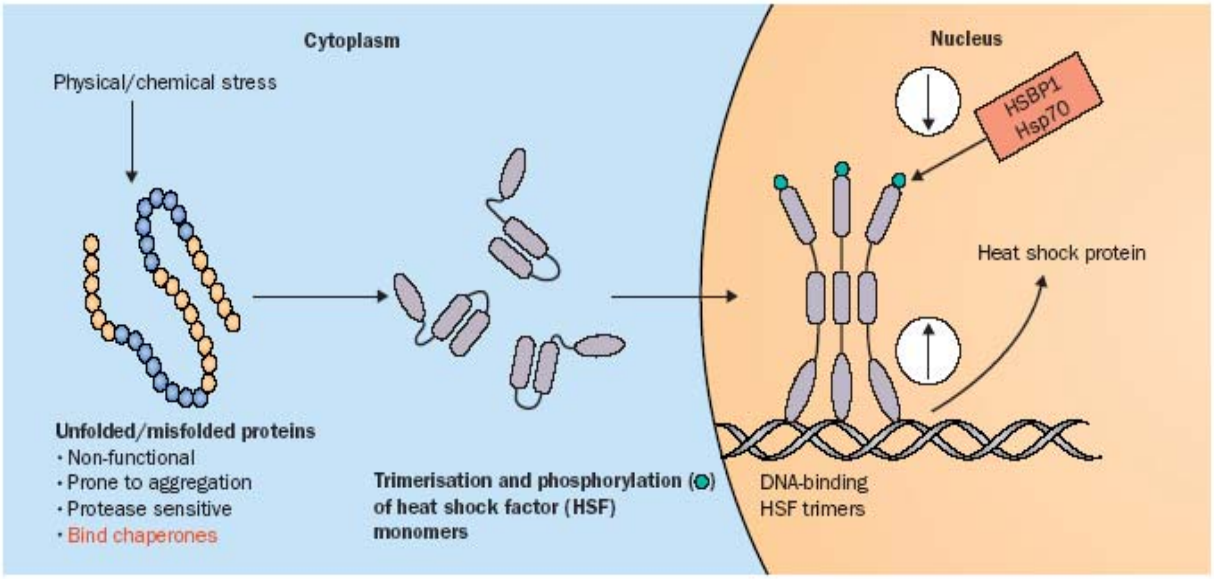

Fig. 2.1. Regulation of heat shock protein gene transcription by heat shock factors (adapted from Pockley, 2003). HSBP1, heat shock binding protein 1; White circles with arrow up, upregulation of Hsp70; White circles with arrow down, downregulation of Hsp70.

In the unstressed state, HSF1 is a latent cytoplasmic protein that is unable to bind DNA. Under stress, HSF1 is hyperphosphorylated in a *ras*-dependent manner by mitogen-activated protein kinases (Knauf et al., 1996, Kim et al., 1997) and consequently converted to phosphorylated trimers that bind DNA. The trimers translocate into the nucleus and interact with Hsp gene promoter regions where they mediate transcription resulting in the eventual synthesis and accumulation of cytosolic Hsps (Johnson and Fleshner, 2006). The activity of HSF trimers is down-regulated by Hsp70 and heat shock binding protein 1 (HSBP1) in the nucleus (Fig. 2.2).

Intracellular Hsp functions

Hsps are constitutively expressed and perform vital functions during protein metabolism and cell maintenance (Lindquist 1986; Morimoto et al., 1994b). As molecular chaperones, Hsps bind the exposed hydrophobic surfaces of non-native proteins denatured by stress (Robert, 2003) and of those not fully synthesized, folded, assembled or localized to the

appropriate cellular compartment (Feder and Hofmann, 1999). By doing so, Hsps maintain other proteins in folding-competent, folded, or unfolded states, contribute to the folding of nascent and altered proteins, assist protein localization, import, and/or export between cell organelles and target non-native or aggregated proteins for degradation and removal from cells.

Extracellular Hsp functions

Increasing evidence suggests Hsps are released into extracellular compartments under certain physiological conditions (Pockley, 2003) and play significant regulatory roles in both innate and adaptive immunity (Phohászka et al., 2002; Robert, 2003). For example, extracellular Hsps are postulated to mediate production of cell surface peptides, thus helping the immune system recognize diseased cells (Moseley, 2000; Deane et al., 2004). Via stimulation of Toll-like receptors TLR2 and TLR4 (Asea et al., 2002; Vabulas et al., 2002), Hsp70 transduces inflammatory danger signals to immune cells such as monocytes, dendritic cells, neutrophils and macrophages (Moseley, 2000; Breloer et al., 2001; Srivastava, 2002a; Fleshner and Johnson, 2005). Hsps also robustly stimulate the secretion of inflammatory cytokines, nitric oxide (NO) synthase, NO, tumor necrosis factor (TNF)-α, Interleukin (IL)-1β and IL-6 from macrophages and neutrophils (Jacquier-Sarlin et al., 1994; Asea et al., 2000; Panjwani et al., 2002; Campisi and Fleshner, 2003; Johnson and Fleshner, 2006). During adaptive immunity Hsps may play an integral part in major histocompatibility complex (MHC) – class II peptide complex assembly (DeNagel and Pierce, 1993) involved in antigen presentation, thus activating T cells to destroy or co-ordinate the killing of pathogens, as well as infected and malfunctioning cells (Srivastava, 2002a, b).

Potential roles of Hsp and their applications

In addition to synthesis during environmental stress, Hsps play substantial roles in plant and animal development (Lindquist and Craig, 1988; Atkinson et al., 1993; Heikkila, 1993). Hsp induction, particularly of Hsp70, extends the *Drosophila* lifespan (Tatar, 1997) and promotes thermotolerance in fish (Basu et al., 2002) and brine shrimp (Clegg et al., 2000a, b). Additionally, stress regulated Hsp accumulation cross-protects against subsequent environmental perturbation (DuBeau et al., 1998; Basu et al., 2002; Rahman et al., 2004). Hsps have potential as bio-indicators of environmental perturbation because they are induced in plants and animals during stress and when exposed to trace amounts of toxins (de Pomerai, 1996; Krasko et al., 1997).

CHAPTER 3

Heat shock proteins in aquatic organisms: Induction, stress tolerance and application[†]

Yeong, Y. S., MacRae, T. H., Sorgeloos, P. & Bossier, P.

[†] References cited in this chapter contain findings presented in subsequent thesis chapters, aiming at drafting a publishable and comprehensive review.

1. Introduction

Heat shock proteins (Hsps), also called stress proteins or molecular chaperones are evolutionary conserved and found in all species of organisms (Lindquist and Craig, 1988). In eukaryotes, these proteins are classified into several groups named primarily on the basis of function, sequence similarities and molecular mass in kilodaltons (kDa). The major Hsp families are Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small heat shock proteins (sHsps) (Lindquist, 1992). Constitutively expressed Hsps, some of which are organelle specific, perform vital functions during metabolism and cell maintenance (Morimoto, 1998). For example, Hsps assist in the folding, and localization of other proteins (Lindquist 1992; Parsell and Lindquist, 1993; Morimoto et al., 1990) and they mediate the intracellular degradation of non-native or aggregated proteins (Feder and Hofmann, 1999). Inducible Hsps, on the other hand, are synthesized under stress and they play crucial roles in the recovery and survival of organisms (Sørenson et al., 2003).

Extensive study of the heat shock response, first discovered by Ritossa (1962), revealed that Hsps are robustly induced by diverse stressors such as heat, cold, hypoxia, starvation, heavy metals, chemicals, crowding, anoxia and disease (Lindquist 1992; Parsell and Lindquist, 1993; Morimoto, 1998). Stressors denature proteins leading to their aggregation, initiation of the cellular stress response and Hsp synthesis (Lindquist 1992). The mechanisms by which stressors stimulate Hsp production converge at transcription where heat shock factors (HSFs) mediate gene expression (Morimoto, 1998). In addition to stress resistance (Iwama et al., 1999; Sun et al., 2002), Hsps are involved in plant and animal development (Atkinson et al., 1993; Heikkila, 1993), aging (Pockley, 2003), environmental adaptation (Koban et al., 1991; Feder and Hofmann, 1999), cross-tolerance (Volker et al., 1992; Dubeau, 1998; Todgham et al., 2005) and the immune response (Srivastava, 2002a, b;

Pockley, 2003; Robert, 2003), demonstrating the fundamental importance of these proteins to cellular well being.

Hsp regulation and function have been examined in the context of aquaculture, with potential applications for these proteins in the production of fish (Dubeau, 1998; Todgham et al., 2005), crustaceans (Clegg et al., 2000a, 2001; Frankenberg et al., 2000; MacRae, 2003) and molluscs (Clegg et al., 1998; Brown et al., 2004). Additionally, there have been several efforts to validate Hsps as stress biomarkers in fish (William et al., 1996; Duffy et al., 1999; Hassanein et al., 1999). However, it has been argued that the use of Hsps in this way is premature because they respond differently to many complex factors including developmental stage, tissue of origin, stressors and season (Iwama et al., 2004). Nonetheless, environmental, physical and biological stressors induce Hsps in aquatic organisms, and a more complete view of their activities may enhance commercial applications. The aim of this review is therefore to illustrate how abiotic and biotic factors regulate Hsp expression in aquatic animals and to describe benefits that accrue from increases in these proteins. Hsp application in aquaculture is also explored, an issue of importance considering the contribution of this technology to feeding the world's population.

2. Hsp induction in aquatic organisms

2.1. Abiotic stressors

2.1.1. Temperature

Rapid temperature shift denatures proteins leading to Hsp induction, and heat stress is the most prominent abiotic stressor in aquatic animals. Heat shock temperatures and the time required to induce Hsp synthesis vary from species to species, and induction depends upon initial acclimation temperatures and the heat tolerance of individual organisms (Table 3.1). Cold water fish species acclimated at 10-16°C up-regulate Hsp expression following heat

shock at 26-30°C. For example, an 8 h heat stress involving an increase in temperature from 10 to 30°C elevates Hsp70 mRNA levels in rainbow trout nucleated red blood cells (Lund and Tufts, 2003), whereas Hsp70 and Hsp90 are significantly augmented in liver and heart of rainbow trout stressed for 1 h with an increase from 13 to 25°C (Rendel et al., 2006). Exposure of green sturgeon larvae to a similar temperature change induces Hsp 70 and 90 (Werner et al., 2007), although 3 days are required. A 15 min increase in temperature from 10 to 14°C promotes Hsp70 synthesis in Atlantic salmon gill and liver (Dubeau et al., 1998), while a 2 h insult from 16 to 22°C boosts Hsp30 and 70 mRNA in the fish torso (Lund et al., 2002). In the latter two studies, a slight temperature change of approximately 5°C induced Hsp in salmon, indicating that this species is relatively sensitive to heat perturbation. This contrasts cold water species such as goldfish, which survive over a broad temperature range, and require a more intense heat shock to elevate Hsps (Kondo et al., 2004; Kagawa, 2004).

Heat shocking warm water fish at 37-40°C after acclimation at 23-27°C up-regulates Hsp expression but this temperature, characterized as lethal for many fish species, may severely compromise stressed animals. In this vein, a 20 h heat shock over similar temperatures induces a two- or three-fold increase in Hsp70 in several Mrigal carp organs (Das et al., 2005). However, prolonged exposure reduces Hsps and causes significant mortalities, demonstrating that excessive heat stress disrupts the Hsp machinery leading to collapse of cellular defense mechanisms and death, as noted by others (Karouna-Renier and Zehr 1999; Tomanek and Somero 1999).

In many aquatic crustaceans, temperatures required to induce Hsp70 production, range from 35-37°C, regardless of the initial acclimation temperature, although the duration of heat stress varies significantly. A 2 h heat shock from 25 to 35°C elevates two Hsp70 genes in the hepatopancrease and thoracic glands of freshwater giant prawn (Liu et al., 2004), while 3 h exposures from 30 to 37°C induce Hsp70 in Monsoon river prawn gill and heart

(Selvakumar and Geraldine, 2005). Heat stress upon increasing temperature from 29 to 35°C for 24 h up-regulates Hsp70 expression in tail muscles of the tiger prawn for up to 8 d (de la Vega et al., 2006). Likewise, the brine shrimp *Artemia* synthesizes proteins typical of other crustaceans experiencing thermal stress. A 30 min heat shock from 21 to 37°C increases constitutive Hsc70 and Hsp67 in adult animals (Clegg et al., 2000a), whereas a change from 28 to 37°C for a similar time up-regulates Hsp70 in larvae (Yeong et al., 2007). These *Artemia* life stages apparently share common heat shock response mechanisms.

Hsp expression after thermal stress has been examined in several oyster species acclimated at 12-18°C, revealing that 70 kDa proteins appear rapidly in gills at 32°C or beyond. Using Pacific oyster as a model, constitutive proteins of 72 and 77 kDa are up-regulated and a 69 kDa protein is induced by a 1 h heat shock, with synthesis occurring both during and after stress (Clegg et al., 1998). Expression of a constitutive 70 kDa protein and an inducible Hsp69 occurs in adult Olympia oysters experiencing similar heat stress (Brown et al., 2004), regardless of season and previous environmental adaptation. In the European flat oyster, heat shock provokes the synthesis of Hsp70 mRNA and protein in gills but not the digestive glands, reaching a maximum after 2 to 3 h of post-stress recovery at 18 °C (Piano et al., 2004, 2005). Unlike the situation in oysters, heat failed to promote accumulation of inducible Hsps in mussels and clams, although constitutive proteins of the 70 kDa family are enhanced significantly (Piano et al., 2004). The expression of Hsp70 may not be a common feature in bivalves undergoing thermal stress.

Low temperature also provokes Hsp expression in fish but research on other aquatic organisms is scarce. Increased Hsp70 expression in channel catfish brain is linked to cold acclimation (Ju et al., 2002). A recent report extends these findings to include muscle tissues, demonstrating that Hsp70 mRNA increases in fish after 14 and 28 days of low temperature incubation (Weber and Bosworth, 2005). The roles and biological ramifications of increasing

Hsp70 have yet to be determined, but these proteins may prevent muscle damage or atrophy in response to environmental stressors, thus protecting fish against chronic cold exposure (Oishi et al., 2003).

2.1.2. Salinity

Exposure to different salinities affects osmoregulation, thus stressing aquatic animals, an effect offset by Hsps. Incubation of Atlantic salmon branchial lamellae, hepatic tissue and erythrocytes in 200–600 mM NaCl, a hyper-osmotic shock, dramatically increases Hsp70 and the 54 kDa stress protein, osp54 (Smith et al., 1999). On the other hand, Hsp90 mRNA accumulates in branchial lamellae upon hyper-osmotic stress *in vivo* (Pan et al., 2000). Kidney exposure to stress like that just described fails to elicit an increase in Hsp90 mRNA suggesting that osmotically induced expression of this protein is tissue specific. Chronic hyper- and hypo-osmotic stresses modulate fish hepatic Hsp60, Hsp70 and Hsp90 (Deane et al., 2002). Black sea bream grown at 33 ppt and adapted long term to iso-osmotic salinity at 12 ppt have low Hsp levels. However, exposure to salinities of 50 and 6 ppt for 8 months increases hepatic Hsp90 2.8 and 2.6-fold, respectively, with Hsp70 and Hsp60 showing similar enhancement under osmotic stress.

Osmotic stresses of 32 to 16 ppt, and 32 to 45 ppt for 30 min respectively elevate Hsp70 and Hsp90 mRNAs in abdominal muscle of American lobster (Spees et al., 2002). In similar experiments, hepatopancreas Hsp90 mRNA increases following hyper-osmotic stress. Conversely, acute hypo-osmotic stress at a much lower salinity of 4 ppt, and hyper-osmotic insults at 150 ppt do not induce Hsp70 in *Artemia* larvae, even though they exhibit reduced body mass (Yeong et al., in press). These observations suggest that crustacean Hsp expression in response to osmotic stress, as for fish is species-specific, not a particularly surprising conclusion when habitat diversity is taken into account.

2.1.3. Environmental contaminants

Linkages between toxicant exposure and cellular Hsps are generally investigated by exposing animals to contaminants in the laboratory or to polluted environmental sites. Such experiments are often employed to test the feasibility of using stress proteins as bioindicators of contaminant exposure and as a measure of ecological status. Heavy metals stimulate Hsp expression in aquatic organisms but this varies greatly from one species to another even though similar stressors are employed. For example, 6 h exposure to river water polluted with copper at 113.9 µg/l and zinc at 348.7 µg/l significantly increases Hsp70 gene transcription in brown trout gills (Hansen et al., 2007). In contrast, shore crabs incubated at a similar copper concentration, even for 2 weeks, only slightly increase gill Hsp70 (Vedel and Depledge, 1995). High zinc levels do not provoke expression of 70 kDa stress proteins in gills of brown mussels, but they do induce accumulation of a 60 kDa stress protein (Franco et al., 2006). Taken together, these studies indicate that Hsp70 is neither a universal nor a sensitive indicator of copper and zinc stress in aquatic organisms, although the protein may be as an indicator for other stressors.

Pesticides increase fish Hsp expression (Sanders, 1993; Hassanein et al., 1999) as shown by contact with pyrethroid esfenvalerate and organophosphate chlorpyrifos which promote Hsp60 and Hsp70 expression in Chinook salmon muscles and gills (Eder et al., 2007). Sub-lethal concentrations of endosulfan promote Hsp70 synthesis in gills of the Monsoon river prawn but not in skeletal muscle and hepatopancreas (Selvakumar et al., 2005). The absence of Hsp70 induction in gills exposed to similar concentrations of carbaryl demonstrates that synthesis is stressor-specific.

Persistent exposure to chlorine and chemical solvents respectively elevates Hsp70 in carp fingerling muscle (Verma et al., 2007) and zebrafish embryos (Hallare et al., 2006). Tributyltin (TBT), a trialkyl organotin compound used in biocides to control a broad

spectrum of organisms, up-regulates Hsp70 in gills of the blue mussel (Pemkowiak et al., 2001). Mercury as CH_3Hg^+, inhibits Hsp70 and induces constitutive Hsc70 synthesis in the Mediterranean mussel digestive gland, whereas Hg^{2+} stimulates Hsp70 and Hsc70 expression (Franzellitti and Fabri, 2006). Fourteen days of contact with arsenic trioxide at 3.8 to 7.6 g/l elevates Hsp70 in liver and kidney of the snake headed murrel (Roy and Bhattacharya, 2006). As opposed to the long exposures and high concentrations required to promote fish Hsp synthesis, a 1 day incubation with 1g/l arsenic substantially induces Hsp70 in bay mussel gill tissues (La Porte, 2005). These studies demonstrate chemically based induction of Hsp70 in fish and bivalves, but effects on other Hsps are yet to be determined. However, hydrogen sulfide up-regulates Hsp60, Hsp70 and several sHsps in surf clams, evidence that chemical insult induces Hsps other than those in the 70 kDa family (Joyner-Matos et al., 2006).

2.1.4. Hormones

Cortisol mediated Hsp expression in fish has been reviewed (Basu et al., 2002), revealing that elevated levels of the hormone suppress Hsp70 induction. The binding of cortisol to glucocorticoid receptors (GR) may displace Hsp70 from mature receptor complexes, increasing free circulating Hsp70, which may then inhibit heat shock transcription factor trimerization and Hsp induction by a negative feedback loop (Basu et al., 2001). However, intraperitoneal implants of 50 µg/g cortisol in rainbow trout upregulates hepatic Hsp90 mRNA, suggesting involvement in GR signaling (Sathiyaa and Vijayan, 2003) and maintenance of tissue responsiveness to cortisol stimulation (Vijayan et al., 2003), possible roles in many organisms (Furay et al., 2006; Brkljacic et al. 2007). GR associates with chaperone complexes that consist of several proteins including p23, Hsp90, 70 and 30, and Hsc70. These complexes bind ligands and chaperone steroid receptors (Pratt, 1997).

Fish injected with recombinant bream GH (rbGH) and ovine prolactin (oPRL), as compared to saline-injected controls, exhibit decreased amounts of hepatic Hsp70 mRNA (Deane et al., 1999). A sea bream fibroblast cell line and primary macrophages incubated with GH at 10 ng/ml experience reduced Hsp70 (Deane et al., 2007). Upon exposure to insulin-like growth factor-I (IGF-1) at 1–10 ng/ml, Hsp70 expression is unchanged in fibroblasts but the protein decreases significantly in macrophages and oPRL at 1–1000 ng/ml decreases fibroblast and macrophage. Clearly, administration of growth hormones and prolactine, either *in vitro* or *in vivo*, down-regulates fish Hsp70 expression. These studies indicate that interactions between Hsps and hormone-mediated physiological responses occur in fish, but to our knowledge, hormone effects on Hsps in aquatic crustaceans and molluscs have yet to be examined.

2.1.5. Handling

Handling does not affect Hsp70 and Hsc71 gene expression in rainbow trout (Vijayan et al., 1997). Similarly, transport and netting fail to induce Hsp60 and Hsp70 in rainbow trout muscle, liver, gills, and heart (Washburn et al., 2002). However, Hsp70 mRNA increases in the European bass during transport (Poltronieri et al., 2007), with inducible Hsp70 mRNA significantly higher in muscle and skin of adults. Future studies are crucial to elucidate relationships between handling and Hsp expression in fish, information important for development of Hsps as biomarkers of stress.

2.2. Biotic stressors

Far less is known about biotic as opposed to abiotic stressors, but various pathophysiological stressors influence Hsp expression. In this context, Hsp70 is augmented significantly when goldfish are reared in the presence of a predator, the bluegill sunfish

(Kagawa et al., 1999). Confinement and crowding increase Hsp70 in tilapia liver, heart and gill (Dini et al., 2006), whereas in sea bass Hsp70, but not Hsp90, mRNA accumulates after crowding (Gornati et al. 2004). Challenge with pathogenic bacteria and viruses promotes Hsp expression in aquatic animals, with *V. anguillarum*, the causative agent of acute vibriosis, inducing Hsp70 in sea bream hepatic tissue (Deane et al., 2004), and Hsp70 mRNA in bay scallop hemocytes (Song et al., 2005). Infection with *V. alginolyticus* promotes Hsp70 mRNA in Mediterranean mussel hemocytes (Cellura et al., 2007). Muscle Hsp70 increases in juvenile Chinook salmon infected with hematopoietic necrosis virus (IHNV), with the highest virus titers accompanied by the strongest Hsp induction (Eder et al., 2007). Collectively, these data substantiate relationships between Hsps and disease in aquatic organisms, suggesting that pathogens damage cell components through release of cytolytic substances, hence altering homeostasis and triggering Hsp induction.

Table 3.1. Factors that influence Hsp production in aquatic organisms

Stressors	Common name (Species)	Stress condition	Hsps induced	Tissues examined	Functional significance	References
Abiotic						
Temperature	Goldfish (*C. auratus*)	4 h heat shock from 20°C to 40°C	Hsp30, Hsp70 mRNA	Cells derived from caudal fin	-	Kondo et al., 2004
		2 h heat shock from 22°C to 32°C	Hsp72, Hsp90	Brain	-	Kagawa, 2004
	Rainbow trout (*O. mykiss*)	8 h heat shock from 10°C to 30°C	Hsp70 mRNA	Red blood cells	Hsp70 may preserve membrane integrity and/or ion regulatory processes.	Lund & Tufts, 2003
		1 h heat shock from 13°C to 25°C with 18-24 h recovery	Hsp70, Hsp90	Liver and heart tissues	Hsp70 and Hsp90 mount a coordinated cellular stress response thereby ensuring a minimum level of protection during thermal stress.	Rendel et al., 2006
	Green sturgeon (*A. medirostris*)	3 d heat shock from 17°C to 26°C at 1.5°C/h	Hsp72, Hsp78, Hsp89	Whole larvae	Hsp72 and Hsp78 are linked to phenotypic variation in the response and vulnerability of larvae to thermal stress	Werner et al., 2007
	Atlantic salmon (*S. salar* parr)	2 h heat shock from 16°C to 22°C and from 25°C and 28°C	Hsp30 mRNA Hsp70 mRNA	Whole body torso	-	Lund et al., 2002
		15 min heat shock from 10-14°C to 26°C	Hsp70	Gill and liver	Confers tolerance to osmotic shock	Dubeau et al., 1998

Stressors	Common name (Species)	Stress condition	Hsp(s) induced	Tissues examined	Functional significance	Reference
	Mrigal carp (*C. mrigala*)	20 h heat shock at 23-27°C to 37°C	Hsp70	Kidney, gill, liver and brain	-	Das et al., 2005
	Channel catfish (*I. punctatus*)	Exposure to low temperature from 25°C to 10.5°C for 14 and 28 d	Hsp70 mRNA	Muscles	-	Weber & Bosworth, 2005
	Monsoon river prawn (*M. malcolmsonii*)	3 h heat shock from 20°C to 32-34°C and 30°C to 36°C-38°C with 1 h recovery	Hsp70	Gill and heart	-	Selvakumar & Geraldine, 2005
	Freshwater giant prawn (*M. rosenbergii*)	2 h heat shock from 25°C to 30°C and 35°C	Hsp70 mRNA	Hepatopancrease and thoracic glands	-	Liu et al., 2004
	Tiger prawn (*P. monodon*)	24 h heat shock from 29°C to 35°C	Hsp70	Tail muscle	Increased Hsp70 may be linked to constant low level of Gill Associated Virus (GAV)	de la Vega et al., 2006
	Brine shrimp (*A. franciscana*)	30 min heat shock from 21°C to 37°C	Hsp70	Whole body (adult)	Induces thermotolerance	Clegg et al., 2000
		30 min heat shock from 28°C to 37°C with 6 h recovery	Hsp70	Whole body (nauplii)	Cross-protects against pathogenic *V. campbellii* and *V.proteolyticus*	Yeong et al., 2007
		1h cold shock from 28°C to 4°C and heat shock to 37°C for 30 min with 6 h recovery	Hsp70	Whole body (nauplii)	Confers protection against pathogenic *V. campbellii* and induces thermotolerance	Yeong et al., (in press)

Stressors	Common name (Species)	Stress condition	Hsp(s) induced	Tissues examined	Functional significance	Reference
	American lobster (*H. americanus*)	2 h heat shock from 15°C to 28°C with 6 h recovery	Hsp90 mRNA	Midgut gland	-	Chang et al., 1999
	Blue mussels (*M. edulis*)	24 h heat shock from 4°C to 20°C	Hsp70	Gill	Confers tolerance to cadmium	Tedengren et al., 2000
	Pacific oyster (*O. gigas*)	1h heat shock from 12°C to 37°C	Hsp70	Gill	Induces thermotolerance	Clegg et al., 1998
	Olympia oyster (*O. conchaphila*)	1 h heat shock from 12-15°C to 33-38°C	Hsp70	Gill	Induces thermotolerance in adults only	Brown et al., 2004
	European flat oyster (*O. edulis*)	1 h heat shock from 18°C to 34°C with 24 h recovery at 18°C	Hsp70	Gill	-	Piano et al., 2004
		Heat shock from 18°C to 35°C with 1 to 6 h recovery	Hsp69 mRNA	Gill	-	Piano et al., 2005
Salinity	Black sea bream (*M. macrocephalus*)	Exposure to chronic hypersaline from 33 ppt to 50 ppt and hypoosmotic from 33 ppt to 6 ppt for 8 months	Hsp60, Hsp70, Hsp90	Hepatic	-	Deane et al., 2002
	Atlantic salmon (*S. salar* parr)	24 h exposure from freshwater to 32 ppt	Hsp90 mRNA	Branchial lamellae	-	Pan et al., 2000

Stressors	Common name (Species)	Stress condition	Hsp(s) induced	Tissues examined	Functional significance	Reference
	Lobster (*H. americanus*)	30 min acute hyper-osmotic stress from 32 ppt to 45 ppt and hypoosmotic shock from 32 ppt to 16 ppt	Hsp70 and Hsp90 mRNAs	Abdominal muscle	-	Spees et al., 2002
		30min hyper-osmotic stress	Hsp90 mRNAs	Hepatopancreas (midgut gland)		
Contaminants	Brown trout (*S. trutta*)	Fish were caged 6 h in a river containing 698 ng/l Cd, 348.7 µg/l Zn and 113.9 µg/l Cu	Hsp70 mRNA	Gill	-	Hansen et al., 2007
	Common carp (*C. carpio*)	28 d whole body exposure to 0.1 mg/l chlorine	Hsp70	Muscles	-	Verma et al., 2007
	Chinook salmon (*O. tshawytscha*)	4 d whole body exposure to 0.08 µg/l pyrethroid esfenvalerate (EV)	Hsp60, Hsp70	Muscle and gills	Hsp appears to be sensitive integrative indicator of stress	Eder et al., 2007
	Snake headed murrel (*C. punctatus*)	14 d whole body exposure to 3.8 g/l and 7.6 g/l arsenic trioxide (50 doses)	Hsp70	Liver and kidney	-	Roy and Bhattacharya, 2006
	Zebrafish (*D. rerio*)	96 h exposure of embryos to 1.5% ethanol, 0.01-0.1% DMSO and 0.1% (v/v) acetone polycyclic	Hsp70	Embryos	Hsp may protect growing embryos from toxic effects of solvents and serves as potential biomarker for teratogenesis	Hallare et al., 2006

Stressors	Common name (Species)	Stress condition	Hsp(s) induced	Tissues examined	Functional significance	Reference
	Rainbow trout *(O. mykiss)*	4 d whole body exposure to 10 µl carbon tetrachloride (CC14) and 100 µl pyrene	Hsp90	Transcriptomes of kidney and liver	-	Krasnov et al., 2005
	Shore crabs (*C. maenas*)	2 weeks whole body exposure to 100 µg copper	Hsp70	Gill	Hsp is not a sensitive indicator of cellular stress	Vedel and Depledge, 1995
	Monsoon river prawns (*M. malcolmsonii*)	48 h whole body exposure to 0.016 and 0.32 µg/l pesticides endosulfan	Hsp70	Gill	Hsp70 is not a general marker of pesticide toxicity	Selvakumar et al., 2005
	Brown mussel *(P. perna)*	48 h whole body exposure to 30 µM zinc	Hsp60	Gill	-	Franco et al., 2006
	Blue mussel *(M. edulis)*	7 d whole body exposure to 50 µg TBT	Hsp70	Gill	-	Pemkowiak et al., 2001
	Mediterranean mussel *(M. galloprovincialis)*	8 h whole body exposure to 0.5 µM mercury	Hsp70 mRNA	Digestive glands	-	Franzellitti and Fabri, 2006
	Bay mussel *(M. trossulus)*	24 h whole body exposure to 100 and 1000 µg/l arsenic	Hsp70	Gill	Hsp70 is not a sensitive biomarker of heavy metal toxicity	La Porte, 2005
	Surf clams *(D. variabilis)*	24 h whole body exposure to 98 ± 2.9 µmol/l hydrogen sulfide	Hsp60, Hsp70, sHsps	Whole body	-	Joyner-Matos et al., 2006

Stressors	Common name (Species)	Stress condition	Hsp(s) induced	Tissues examined	Functional significance	Reference
Hormones	Rainbow trout (*O. mykiss*)	Intraperitonial implant with 50 mg cortisol/kg	Hsp90 mRNA	Liver	Hsp90 is involved in the upregulation of glucocorticoid (GR) mRNA by cortisol	Vijayan et al., 2003
Handling	European bass (*D. labrax*)	3 h transport stress	Hsp70 mRNA	Skin and skeletal muscles	-	Poltronieri et al., 2007
Biotic						
Crowding	Tilapia (*O. mossambicus*)	4-5 h of confinement and crowding	Hsp-70	Liver and heart	Hsp70 may be indicator of crowding stress	Dini et al., 2006
Disease	Chinook salmon (*O. tshawytscha*)	20 d exposure to 6.4 x 10^5TCID/50ml infectious hematopoietic necrosis virus (IHNV)	Hsp70	Muscle	-	Eder et al., 2007
	Silver sea bream (*S. sarba*)	Intramuscular injection with 4.9 x 10^4 cfu/fish of live *Vibrio alginolyticus*	Hsp70	Hepatic	-	Deane et al., 2004
	Flounder (*P. olivaceus*)	*In vitro* exposure to UV-inactivated Turbot (*Scophthalmus maximus L.*) rhabdovirus (SMRV)	Hsp40 mRNA	Embryonic cells	Hsp40 may function as co-chaperone with Hsp70 in immune response	Dong et al., 2006
	Bay scallop (*A. irradians*)	Injection with 50 µl (2 x 10^8 cells/ml) of live *V. anguillarum* into adductor muscles	Hsp70 mRNA	Hemocytes	Hsp mediates environmental stress and immune response in scallops	Song et al., 2005
	Mediterranean mussel (*M. galloprovincialis*)	Injection with 100 µl (1 x 10^7 cells/ml) of heat killed *V. anguillarum* into adductor muscles	Hsp70 mRNA	Hemocytes	-	Cellura et al., 2007

3. Induced thermotolerance in aquatic organisms

Thermotolerance in living organisms is dependent upon Hsp production in response to an initial heat stress, and in some cases Hsp translocation between cellular compartments is also required (Kampinga et al., 1995). Frequently, the pre-induction protocol to render an organism thermotolerant entails two phases, namely a short induction by an acute heat shock followed by several hours of expression under non-stress conditions. Enhanced thermotolerance in fish after Hsp expression has been reviewed extensively by Iwama et al., (1999) and Basu et al., (2002). This phenomenon is not, however, restricted to fish but extends to other animals, indicating that many aquatic organisms acquire thermotolerance by up-regulating endogenous Hsps. For example, a 1 h sub-lethal heat shock from 28 to 40°C induces Hsp70 in larvae of the brine shrimp *Artemia*, and thermotolerance reaches maximum levels in 4 h (Miller and McLennan, 1988a). Heat shock from 22 to 37°C for 30 min with 1 h of recovery induces Hsc70 and Hsp67 and generates thermotolerance in adult *Artemia* for at least 3 days (Clegg et al., 2000a). Moreover, Hsp70 accumulation as a result of a combined hypo- and hyperthermic stress protects gnotobiotic brine shrimp larvae against lethal heat stress (Yeong et al., in press).

Additionally, studies on molluscs suggest a link between induced thermotolerance and Hsps, with heat shock from 12 to 37°C for 1 h inducing constitutive isoforms of 72 and 77 kDa and a 69 kDa protein in the Pacific oyster (Clegg et al., 1998). These animals are then able to survive a normally lethal heat treatment of 43-44°C and thermotolerance is retained for at least 2 weeks. Thermotolerance is attained in the California native oyster by up-regulating constitutive and inducible Hsp70 (Brown et al., 2004). Survival of adults is markedly increased in this case, conferring almost total protection to lethal temperatures. Hsp70 appears to have a crucial role in thermotolerance, an observation commonly made for fish and terrestrial animals.

The inhibition of Hsp70 accumulation prevents thermotolerance development in rainbow trout fibroblasts (Mosser and Bols, 1988). Direct evidence for the role of Hsp70 in heat shock survival came by inhibiting Hsp70 translocation into rat cell nuclei by antibody microinjection, a procedure that reduces survival after even short incubation at lethal temperature (Riabowol et al., 1988). Nuclear translocation of Hsps may protect transcriptional processes following heat stress (Langer et al., 2003; Rendell et al., 2006). Though the stress sensor and the regulation of Hsp induction are not understood, it is clear that Hsp70 plays a major role in coping with high temperature damage. Proteins are repaired and refolded (Hightower et al., 1999; Hofmann, 1999), and soluble proteins are protected against heat denaturation (Jinn et al., 1989), both vital actions in maintaining cellular homeostasis.

Though it is almost certain that Hsp70 mediates thermotolerance, the role of other stress proteins in this process has received little attention for aquatic organisms. Heat shock induces several different insect stress proteins and chaperone complexes (Neal et al., 2007), indicating an influence on thermotolerance. In *Drosophila*, elevated levels of sHsps 22–28 and Hsp70 fail to promote thermotolerance when Hsp90 activity is repressed by ansamycins, suggesting a multicomponent protein chaperone complex establishes thermotolerance (Duncan, 2005). Additionally, Hsp27 and not Hsp70 is associated with thermotolerance in human pancreatic tumor cell lines (Ng et al., 2002). Besides the significant role of Hsps other than Hsp70, it is noteworthy that constitutively expressed, as well as induced stress proteins, influence thermotolerance, which in most cases, requires cooperation between both sets of proteins. Further work is required to elucidate mechanisms underlying the relationship between thermotolerance and Hsp isoforms, and to resolve how their production assists aquatic organisms in responding to lethal heat challenge. Such studies are important

fundamentally and they will assist in formulation of strategies to protect aquatic organisms against thermal stresses, this of particular importance in aquaculture.

4. Cross-tolerance in aquatic organisms

Cross-tolerance, often referred to as cross-protection, entails increased tolerance to physiological insult following an initial transient, albeit different, stress, an ability frequently occurring concomitantly with Hsp accretion. Study of cross-protection in fish was initiated by the demonstration that heat stress shields winter flounder cells against deleterious chemical exposure, an abiotic stress (Brown et al., 1992). Cross-tolerance coincided with Hsp28, Hsp70 and Hsp90 up-regulation. In another example, thermal shock at 26°C for 15 min confers protection against osmotic shock at 45 ppt in salmon (Dubeau et al., 1998), a situation where smolts exhibiting elevated branchial and hepatic Hsp70 consistently display highest survival. In tide pool sculpin, a heat shock of approximately 12°C followed by 4 to 48 h of recovery enhances protection against osmotic and hypoxic stresses, with survival increasing from 68% to 96%, and 47% to 76%, respectively. The strongest association between Hsp70 quantity and cross-tolerance for this species is observed in gills (Todgham, 2005). Hsp70 generated by heat shock cross-protects blue mussels against cadmium (Tedengren et al., 1999). These studies jointly indicate that aquatic organisms acquire enhanced protection against environmental change by regulating endogenous Hsps. Stress induced Hsps, and in particular Hsp70, mediate cross-protection at both cellular and higher organizational levels, and in many different organisms including bacteria (Flahaut et al., 1997; García et al., 2001) plants (Song et al., 2004), insects (Sejerkilde et al., 2003) and terrestrial animals (Arieli et al., 2002; Malyutina et al., 2005). The mechanism by which Hsps initiate cross-protection to abiotic stressors is uncertain, but may require refolding of damaged proteins or binding to irreversibly damaged proteins in order to signal their degradation. Both activities prevent

intracellular accumulation of abnormal proteins and the formation of insoluble aggregates (Parsell and Lindquist, 1993).

Stress regulated Hsps may also bestow cross-tolerance to biotic stressors in aquatic animals. Exposure to a non-lethal heat stress from 28-37°C for 30 min followed by 6 h of recovery shields gnotobiotic *Artemia* larvae against infection by *Vibrio campbellii* and *V. proteolyticus* (Yeong et al., 2007). Larvae given a preconditioning heat treatment which induces Hsp70 acquisition are better protected from challenge by pathogenic bacteria, with survival augmented two-fold as compared to control animals. Furthermore, a combined hypo- and hyperthermic shock followed by a 6 h recovery at ambient temperature contributes similar protective effects (Yeong et al., in press). In this case, gnotobiotic *Artemia* larvae experiencing reduced mortality and lower bacterial load upon *V. campbellii* challenge are enriched in Hsp70. In both studies, Hsp involvement in generating inducible stress resistance to bacterial challenge implies a role for Hsps in shrimp immunity, as discussed later. The involvement of Hsp in induced cross-tolerance to abiotic and biotic perturbations denotes stress protein interaction with multiple molecular and cellular systems in diverse ways. Understanding how stress-regulated Hsps confer cross-tolerance is therefore vital in devising strategies to prime or enhance tolerance in aquatic organisms.

5. Immune enhancement in aquatic organisms

Hsps modulate innate and adaptive immunity (Srivastava, 2002a), and interest in their immunoregulatory roles has fueled research on aquatic organisms. For example, Hsp70 build up after short-term hyperthermic stress correlates with attenuation of gill-associated virus (GAV) replication in the black tiger prawn, *Penaeus monodon* (de la Vega et al., 2000). Endogenous Hsp70 accumulation enhances the resistance of gnotobiotic *Artemia* larvae to *V. campbellii* and *V. proteolyticus* (Yeong et al., 2007; in press). These studies suggest that Hsps

stimulate the innate immune system, thus promoting recognition and subsequent pathogen destruction by defensive mechanisms, results similar to those observed with other organisms. For example, stress regulated sHsps and Hsp90 trigger immunity against *Pseudomonas aeruginosa* in *Caenorhabditis elegans* by a conserved pathway involving heat shock transcription factor-1 (HSF-1) and the associated DAF2/DAF16 pathway (Singh and Aballay, 2006). Evidence for antagonizing effects on virus protein synthesis by high levels of Hsp70 in mammalian cells infected with rhabdovirus also suggests a role in controlling virus replication (Santoro et al., 1994). Hsp70 induction as a result of heat shock may be a key step in the inhibition of viral replication (Demarco and Santoro, 1993; Virgilio et al., 1997; Conti et al., 1999).

Immune stimulation through activation of Toll-like receptors potentially explains how Hsps attenuate shrimp infection. The toll-like receptor from white shrimp, *Litopenaeus vannamei*, contains the distinct structural and functional motifs indicative of this protein family. These include an extracellular domain with 16 leucine-rich repeats (LRRs) flanked by cysteine-rich motifs and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, the latter having 59.9% and 54.3% identity respectively to *Apis mellifer* and *Drosophila melanogaster* Toll (Yang et al., 2007). Characterization of a novel cDNA from gills of the giant tiger shrimp, *Penaeus monodon* reveals that the amino acid sequence of the Toll (PmToll) protein from this organism is 59% similar to an *A. mellifera* Toll-related protein (Arts et al., 2007). Insect and mammal Hsps may activate Toll-like receptors which then transduce inflammatory signals to innate immune cells and promote resistance against disease and infections (Asea et al., 2002; Vabulas et al., 2002: Gobert et al., 2003). It is important to determine if Hsps activate Toll-like receptors in aquatic organisms and consequently mediate the innate immune system.

Hsps may trigger immune responses in diverse ways. Extracellular Hsp70, in combination with other Hsps, could act as a signaling molecule, modulating peptide position on cell surfaces and enhancing immune recognition of aberrant cells (Athman and Philpott, 2004; Johnson and Fleshner, 2006). Hsp60, Hsp70, and Hsp90 potentially chaperone antigenic peptides via the major histocompatibility complex class I presenting pathway and stimulate inflammatory cytokine production (More et al., 2001). Extracellular Hsp70 increases production of inducible nitric oxide synthase and nitric oxide (Panjwani et al., 2002; Campisi and Fleshner, 2003), tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 (Asea et al., 2000; Campisi and Fleshner, 2003) in macrophages and neutrophils (Johnson and Fleshner, 2006). Furthermore, phagocytes and granulocytes release lysozyme, reactive oxygen species, and cationic peptides upon Hsp exposure (Jacquier-Sarlin et al., 1994; Basu et al., 2002). These observations support the hypothesis that Hsps mediate immune responses which robustly suppress infection, but the limited research on this subject for aquatic organisms obscures associations between Hsps, the immune system and disease. Understanding these relationships is necessary to inform establishment of strategies to protect living organisms, particularly in aquaculture where infection and disease are major obstacles to increasing food production.

6. Hsp significance in aquaculture and future perspectives

The temperature within aquatic environments may change substantially during the day and this, coupled with seasonal fluctuations, presents challenges for resident organisms such as the commercially important oysters and salmonid fish which often tolerate these fluctuations poorly (Clegg, et al. 1998; Rendell et al., 2006). Consequently, mass mortalities occur in the field, resulting in large financial losses for farmers. Ectothermic aquaculture species may also exhibit inadequate survival following transfer from hatcheries to the field,

perhaps due to abrupt temperature shift. Osmotic shock is severe in commercial salmon culture when juveniles are transferred directly from freshwater to seawater, this contrasting the gradual transition to full salinity seawater afforded under natural conditions (Dubeau et al., 1998). Hsp ability to confer resistance against many stresses can alleviate these types of problems. Hsp accumulation induced by heat shock could prime aquatic organisms against environmental perturbations, allowing them to survive extreme conditions. Induced Hsp synthesis might improve larval performance during transport and under harsh culture conditions. A sub-lethal heat shock is possibly not the best way to enhance Hsp production because acute temperature shifts are often detrimental, causing significant mortalities and adversely affecting physiological balance. Less traumatic approaches to up-regulate Hsp expression in aquatic organisms are required.

That endogenous Hsp accumulation confers resistance against pathogenic challenge may allow priming of shrimp against disease and infections. Hsps arise as dominant antigens in many microbial infections, rendering these pathogen-derived proteins potentially useful for fish vaccination. Characterization of a novel immunogenic protein synthesized by *Piscirickettsia salmonis* obtained from infected salmonid fish revealed a prokaryotic Hsp, supporting the idea that bacterial Hsps are useful for vaccine design (Marshall et al., 2007). Moreover, vaccination using bacterial Hsps induces significant protection in terrestrial animals. For example, recombinant DnaJ from *Salmonella enterica serovar* Typhi, shields mice against infection by *S. typhimurium* (Sagi et al., 2006) and Hsp60 from *Histoplasma capsulatum* increases mouse resistance to pulmonary histoplasmosis (Gomez et al., 1995). Uncertainty regarding relationships between Hsps and the immune system hinders Hsp vaccine use in fish. Hence, future research must focus on this to exploit an intriguing approach for control of disease in fish and other commercial organisms.

Administration of prokaryotic Hsps in different ways confers disease tolerance in shrimp. Feeding with bacteria over-producing prokaryotic Hsps, particularly DnaK, either following heat shock or upon induction after transformation with heterologous cDNA, boosts *Artemia* survival approximately three-fold upon challenge with pathogenic *V. campbellii* (Yeong et al., unpublished). The mechanism by which Hsp producing bacteria protect brine shrimp against *Vibrio* infection has yet to be determined, but immune stimulation is possible. This observation has applied significance in aquaculture where disease associated with luminescent vibrios kills cultured organisms, causing economic loss (Diggles et al., 2000; Austin and Zhang, 2006). Antibiotic therapy is used but this is costly, resulting in microbial resistance, tissue accumulation of antibiotic residues (Vadstein, 1997), and immuno-suppression (Hameed and Balasubramanian 2000; Smith et al., 2003), all serious threats to human health. Hence, Hsps are potentially an alternative to antibiotics as a way to boost the resistance of aquaculture organisms to disease and infection.

Hsp application in aquaculture is still in its infancy, but accumulating evidence demonstrates that these proteins play important roles in aquatic organism growth and survival, ranging from stress tolerance to immune enhancement. How aquatic organisms benefit from elevated Hsps remains an important question. Functional genomic approaches are crucial for elucidating the complex and integrative regulatory mechanisms of Hsps and their actions in aquatic organisms.

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CHAPTER 4

Non-lethal heat shock protects gnotobiotic *Artemia franciscana* larvae against virulent Vibrios

Yeong, Y. S., Van Damme, E. J. M., Sorgeloos, P. & Bossier, P.

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Abstract

Brine shrimp *Artemia* were exposed under gnotobiotic conditions to a non-lethal heat shock (NLHS) from 28 to 32, 37 and 40°C. Different recovery periods (2, 6, 12 and 24 h) and different heat-exposure times (15, 30, 45 and 60 min) were tested. After these NLHS, *Artemia* was subsequently challenged with *Vibrio*. Challenge tests were performed in stressed and unstressed nauplii at concentrations of 10^7 cells/ml of pathogenic bacteria, *Vibrio campbellii* and *Vibrio proteolyticus*. A NLHS with an optimal treatment of 37°C for 30 min and a subsequent 6 h recovery period resulted in a cross-protection against pathogenic *Vibrio*. A 100% increase in the larval survival ($p<0.05$) was observed. We have also demonstrated by Western blot that a NLHS increases the expression of Hsp70 in heat-shocked (HS) treated animals. This report is the first to reveal a cross protection of a NLHS against deleterious bacterial challenges in living crustaceans. The putative role of heat shock proteins (Hsps) in this process is discussed.

Introduction

Over the years, the brine shrimp *Artemia* has gained popularity as the most widely used live diet in fish and shellfish larviculture. Different *Artemia* species, found in harsh environments worldwide (Triantaphyllidis et al., 1998; Van Stappen et al., 2002; Tanguay et al., 2004), have been proven to be useful model organisms for stress response studies (Clegg et al., 2002). The wide range of stress tolerance has made this unique genus to be termed recently as the '*animal extremophile*' (Clegg, 2005).

Heat shock involves the sudden exposure of cells, tissues and organisms to a temperature well above the ambient condition but still within the physiological range of the organism (Rahman et al., 2004). In almost all organisms, heat shock will activate the transcription and synthesis of a specific group of proteins known as the heat shock proteins (Lindquist, 1992). Some of these Hsps are thought to act as molecular chaperones whereby they assist in the recovery of misfolded or aggregated proteins and protect organisms against extreme forms of stress (Parsell and Lindquist, 1993; Morimoto et al., 1994; Feder and Hofmann, 1999). Hsps are not induced solely by heat shock. Expression of these proteins are also up-regulated by various physiological perturbations such as oxidative stress, nutritional deficiencies, ultraviolet radiation, chemicals, ethanol, viral infection and anoxia (Rahman et al., 2004; Pockley, 2003).

Until recently, many research on stress response aimed at understanding the localization, structures and specific functions of intracellular Hsps (Parsell and Lindquist, 1993; Feder and Hofmann, 1999, Feige and Mollenhaure, 1992). Hsps are involved in cross-protection or cross-tolerance in animals and plants, i.e. a general stress response and a transient increase in the resistance to a second heterologous physiological and environmental insult (Volker et al., 1992). Just to select a few important reviews from a massive body of literatures, an initial heat stress confers thermal resistance (Frankenberg et al., 2000; Periago

et al., 2002; Sejerkilde et al., 2003; Lei et al., 2005), protect against osmotic stress (Volker et al., 1992; Jenkins et al., 1990; DuBeau et al., 1998; Todgham et al., 2005; Neta et al., 2005), prevent oxidative toxicity and damage (Todgham et al., 2005; Arieli et al., 2003; Collins and Clegg, 2004) and improve desiccation tolerance (Ma et al., 2005). These observation clearly illustrate that heat stress protects multiple organisms against a further and eventually, more severe environmental insults. However, there is limited information on the effect of a NLHS on the subsequent resistance of the host against pathogens, probably because it is experimentally difficult to apply a heat treatment to a host without affecting the associated microbial community, either in its composition or its physiology.

The *Artemia* gnotobiotic experimental system is an excellent model system to control any interference of microbes during a NLHS and thus offering full experimental control (Marques et al., 2004a). The present study aims at investigating the relationship between a NLHS and its subsequent cross protection against pathogenic bacteria. Gnotobiotic *Artemia* nauplii were exposed to several heat shock conditions and subsequently challenged with *V. campbellii* (LMG21363) and *V. proteolyticus* (CW8T2). The *Vibrio* strains were previously described as relatively strong pathogenic bacteria for *Artemia* (Soto-Rodriguez et al., 2003; Verschuere et al., 2000b; Marques et al., 2006a). Detection of Hsp70 expression at different NLHS temperatures was performed using SDS-PAGE and Western immunoblotting.

Materials and methods

Bacteria culture

Isolates of the bacterial strains, *V. campbellii* (LMG21363) and *V. proteolyticus* (CW8T2), previously stored in 40% glycerol at –80°C, were aseptically inoculated and grown in Petri dishes containing marine agar 2216. A colony was subsequently transferred and grown to stationary phase in marine broth 2216 (Difco Laboratories, Detroit, Mich.) by

incubation overnight at 28°C with constant agitation. Bacterial suspensions were then transferred to centrifugation tubes and centrifuged at 2200 g for 15 min. The supernatant was discarded and pellets were resuspended in filtered autoclaved sea water (FASW). The bacterial densities were determined spectrophotometrically at an optical density of 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/ml.

Axenic *Artemia* culture

Axenic *Artemia* were obtained following decapsulation according to Sorgeloos et al., (1986) and hatching procedures described by Marques et al. (2004a). Bacteria-free cysts and nauplii were obtained via decapsulation whereby the hard shell called chorion that encysts the dormant *Artemia* embryo is completely removed by short-term exposure to a hypochlorite solution. High-hatching cysts of *Artemia franciscana*, originating from the Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium) were used. About 1g of cysts were hydrated in 90ml tap water for 1 h with strong aeration in non-axenic conditions. The recipient with the cysts was then transferred to a laminar flow hood where decapsulation procedures were performed. All equipment was previously sterilized and autoclaved at 120°C for 20 min prior to use. A 0.22µm-filtered aeration was provided to avoid bacterial contamination. Then, 50 ml of cold sodium hypochlorite containing 15% (w/v) active chlorine and 3.3 ml of 32 % (w/v) sodium hydroxide were added to the hydrated cysts. The reaction was stopped after 150s by adding 70ml of autoclaved sodium thiosulphate pentahydrate (10 mg/l). Decapsulated cysts were washed several times with FASW and collected over a 50µm sterile sieve. A few mg of these cysts was subsequently transferred to separate, sterile 50ml Falcon tubes containing 30 ml of FASW and carefully capped. For hatching incubation, the tubes were placed on a rotor at 4 cycles per min and constantly exposed to incandescent light (± 41µEm^{-2}) at 28°C for 18-24 h. Consequently, hatched

nauplii that developed into stage II within the next 4-6 h were used in the experiments (only in stage II, the nauplii´s mouth opens, allowing ingestion of *Vibrio*).

Heat shock preparation and *Artemia* larvae collection

For hyperthermic treatment, *Artemia* nauplii kept in 200ml of FASW at 28^{o}C with an approximate density of 5 animals/ml were exposed to a NLHS at a Δt rate of 2°C/min in a preheated and controlled water bath system with thermostat heaters accurate at ± 0.5°C. Heat-shocked *Artemia* were slowly acclimatized back to a water temperature of 28°C (t = 0.5°C/min). Various recovery periods were tested prior to pathogen challenge. Nauplii were then picked and transferred into a Petri dish by using sterile Pasteur pipettes. Fifty stage II *Artemia* nauplii were subsequently counted and picked into sets of Falcon tubes, which were previously filled with 30 ml FASW and incubated at 28°C. Three replicates were prepared for each treatment and bacteria free status was verified in the blanks. Larvae kept at 28°C were used as controls in all experiments. All procedures were aseptically performed in a laminar flow hood.

Axenity verification

Methods used to verify axenity of the *Artemia* culture were conducted following (Marques et al., 2004a) by using a combination of plating and live counting techniques. Bacterial contamination in the control tubes was checked by plating 100 µl of the culture medium in marine agar. Plates were then incubated for 5 days at 28°C. Live counting involved staining of the blanks with tetrazolium salt MTT (-3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 0.5% w/v) in a sterile recipient (1 part of MTT to 9 parts of sample) incubated at 30°C for 30 min. Live bacterial detection and counting were

performed using a light microscope at 1000 x magnification. If contaminated control tubes were found, all results of that experiment were discarded.

Experimental design and challenge test

In the first experiment, the intensity of the NLHS was tested by exposing axenically-hatched *Artemia* nauplii to a series of different HS temperatures from 28 to 32, 37 and 40°C for 30 min, with a subsequent recovery period of 6 or 24 h. In all experiments, non-HS nauplii were used as control. Challenge tests performed in this experiment were conducted following a small modification of the technique described by Marques et al., (2006a) i.e. the bacterial suspensions containing isolates of pathogenic *V. campbelii* were added at concentrations of 10^7 cells/ml. All tubes were incubated under constant agitation and light. Nauplii were not fed throughout the experiment. *Artemia* survival was determined after 24 h of exposure by counting the remaining live nauplii. The best NLHS temperature (with the highest *Artemia* larval survival) was selected to perform the subsequent experiments.

Experiment 2 involved testing the effect of different recovery periods in the *Vibrio* challenge test. Axenically-hatched *Artemia* nauplii were given a NLHS at 37°C (best temperature selected from previous experiments) for 30 min. The recovery periods tested were 2, 6, 12 and 24 h of incubation at 28°C before starting the challenge test. The optimal recovery period was chosen and used in subsequent experiments. In experiment 3, the duration of NLHS was investigated namely 15, 30, 45 and 60 min, respectively. The animals were given a 6 h recovery period (best recovery period selected from experiment 2) and subsequently challenged with 10^7 cells/ml of *Vibrio campbellii*. Larval survival was determined 24 h after challenge.

In the last experiment, *Artemia* nauplii exposed to optimal NLHS conditions were challenged with 10^7 cells/ml of *V. campbellii* and *V. proteolyticus*. All experiments were

performed twice to verify the reproducibility of the results and each treatment was performed in three replicates.

Protein Extraction, SDS-PAGE and Western blot

Hsp70 was analyzed in the controls and in heat-shocked nauplii under optimal NLHS conditions. Protein extraction was performed essentially as described by Frankenberg et al., (2000). HS and non-HS treated *Artemia* nauplii were collected on cloth filters and rinsed with ice-cold distilled water to remove external sea water. About 200mg wet weight tissue ml^{-1} (equal amount of animals in all treatments) were homogenized in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4), pre-added with a protease inhibitor cocktail (Sigma-Aldrich, Inc. USA) at recommended level of 1:10 of extracts. Aliquots of homogenate were then combined with equal volumes of 2 x SDS sample buffer, vortexed and heated at 95°C for 5 min (Laemmli, 1970). Samples were cooled and centrifuged at 1630 *g* for 3 min to remove chitinous exoskeleton fragments that prevented accurate pipetting. Protein concentrations of each sample were measured according to the Bradford method. Protein concentration was calculated and equal amounts of protein were loaded on the gels enabling direct comparison between different samples in the gel and Western blot. Western blot on equal aliquots was tested as well.

Protein extracts were electrophoresed on 15% poly-acrylamide gels. Two gels were run simultaneously: one was stained with Coomassie brilliant blue and the other transferred to a polyvinylidene fluoride transfer membrane (Biotrace^{TM} PVDF, TALL, Gelman Laboratory, USA) for Western immunoblot analysis. The membrane was blocked overnight at 4°C using blocking buffer (phosphate buffer saline + Tween-20 + 5% bovine serum albumin) and subsequently incubated with mouse monoclonal anti-Hsp 70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO) at recommended dilutions of 1:5000. This

antibody detects both constitutive and inducible isoforms of the Hsp70 family. Polyclonal rabbit anti-mouse IgG (Dako®, Denmark) with horseradish peroxidase conjugate was used as secondary antibody at recommended dilutions of 1:1000. For detection, 0.7 mM diaminobenzidinetetrahydrochloride dihydrate (DAB) was used as a substrate in association with 0.01 % (v/v) H_2O_2 in 0.1 M Tris-HCl (pH 7.6).

Data collection and analysis

After challenge, larval survival was determined in all experiments by counting the actively swimming animals. Live nauplii were picked and fixed in Lugol's solution to facilitate counting. Values of larval survival (%) were ArcSin-transformed to satisfy normal distribution and homocedasticity. Differences between HS and non-HS treated larval survival in the *Artemia* challenge tests were investigated by performing analysis of variances (ANOVA) using statistical analysis software SPSS® version 11.5 for Windows®.

Results

Heat shock temperatures

Axenic *Artemia* nauplii that were given a NLHS followed by a recovery period of 6 h at 28°C performed better in the challenge test (Table 4.1). Survival of nauplii was significantly higher ($p<0.05$) in all NLHS treatments, namely HS32, HS37 and HS40, as compared to the controls. However, the HS40 treatment in the absence of challenge caused high larval mortality (data not shown) and therefore was not considered to be suitable. High mortality was also observed in the challenge test when nauplii were given a NLHS followed by a 24 h recovery period most probably due to starvation. Based on these results, it was concluded that a HS from 28°C to 32°C for 30 min would be sufficient to boost protective

effects of *Artemia* nauplii towards resistance against pathogenic bacteria, but HS37 (with the highest survival rates) was subsequently chosen for experiments 2 and 3.

Table 4.1 - Average survival (%) of *Artemia* nauplii after 24 h challenge test using 10^7 cells/ml of *V. campbellii* in relation to different heat shock (HS) temperature for 30 min exposure with subsequent 6 h and 24 h recovery periods (Experiment 1).

HS treatments (^{O}C)	A		B	
	Survival (%)		Survival (%)	
	6h recovery	24h recovery	6h recovery	24h recovery
CTR 28	36 ± 4^{a}	14 ± 5^{a}	38 ± 6^{a}	17 ± 6^{a}
HS 32	65 ± 2^{b}	17 ± 4^{a}	63 ± 5^{b}	18 ± 5^{a}
HS 37	70 ± 7^{b}	28 ± 9^{a}	71 ± 2^{b}	24 ± 5^{a}
HS 40	68 ± 8^{b}	27 ± 10^{a}	63 ± 1^{b}	21 ± 8^{a}

For each average, the respective standard deviation is added (mean ± S.D.). Each experiment was repeated twice: A and B. CTR – control without heat treatment (28°C); HS – heat shock. Values in the same column (for each experiment) showing the same superscript letter are not significantly different ($p>0.05$).

Recovery periods

Artemia nauplii that were given a recovery period of 2 h and 6 h after heat treatment showed significantly higher survival rates ($p<0.05$) in the challenge test as compared to the non-HS controls (Table 4.2). Larval survival declined after prolonged recovery periods in both HS and the controls treatments (CTR). A 6 h recovery period (with the largest difference in larval survival between HS and CTR) was chosen for experiment 3.

Table 4.2 - Average survival (%) of *Artemia* nauplii after 24 h challenge test using 10^7 cells/ml of *V. campbellii* in function of different recovery periods with non-lethal heat shock temperature of 37°C for 30 min (Experiment 2).

Recovery period (hours)	A		B	
	Survival (%)		Survival (%)	
	CTR	HS	CTR	HS
2h	33 ± 3^{a}	$65 \pm 5^{a,*}$	37 ± 2^{a}	$60 \pm 2^{a,*}$
6h	29 ± 5^{a}	$63 \pm 6^{a,*}$	30 ± 6^{a}	$65 \pm 6^{a,*}$
12h	24 ± 4^{a}	$45 \pm 5^{b,*}$	25 ± 5^{a}	$43 \pm 2^{b,*}$
24h	9 ± 4^{b}	$30 \pm 2^{c,*}$	13 ± 1^{b}	18 ± 7^{c}

For each average, the respective standard deviation is added (mean ± S.D.). Each experiment was repeated twice: A and B. CTR – control without heat treatment (28°C); HS – heat shock. Values in the same column (for each experiment) showing the same superscript letter are not significantly different ($P>0.05$). * indicates significance different between HS and control (non-HS) treatments ($p<0.05$).

Duration of heat shock

Significant differences ($p<0.05$) in larval survival were observed in the challenge test between HS nauplii and the control (Table 4.3). However, data revealed that the duration of the HS does not have a big impact on survival enhancement although a 15 min NLHS might be the minimum. A 30 min NLHS (resulting in the highest survival) was chosen for the following experiments.

Table 4.3 - Average survival (%) of *Artemia* nauplii after 24 h challenge test using 10^7 cells/ml of *V. campbellii* in function of different heat shock duration with non-lethal heat shock temperature of 37°C and 6 h recovery period (Experiment 3).

HS duration (minutes)	A	B
	Survival (%)	Survival (%)
CTR	36 ± 4^{c}	33 ± 3^{c}
15 min	67 ± 10^{b}	64 ± 4^{b}
30 min	77 ± 6^{a}	74 ± 5^{a}
45 min	76 ± 4^{ab}	71 ± 8^{ab}
60 min	74 ± 9^{ab}	65 ± 5^{ab}

For each average, the respective standard deviation is added (mean ± S.D.). Each experiment was repeated twice: A and B. CTR – control without heat treatment (28°C); HS – heat shock. Values in the same column (for each experiment) showing the same superscript letter are not significantly different ($p>0.05$).

Challenge test at optimal NLHS condition

HS treated *Artemia* nauplii were always better protected in the challenge test. Survival more or less doubled by applying a NLHS (37°C, 30 min, and 6 h recovery). Significant differences in survival rates (p<0.05) were observed between controls and HS treated *Artemia* in both challenge tests (*V. campbellii* and *V. proteolyticus*) (Fig. 4.1A, B). Data obtained showed that the overall survival of both the controls and HS treated nauplii were slightly higher when challenged with *V. proteolyticus* as compared to *V. campbellii*, suggesting that *V. proteolyticus* is less virulent. Both the negative control treatments, which consisted of unchallenged HS and non-HS animals recorded no larval mortalities and insignificant differences (p>0.05) in the larval survival (data not shown), thus verifying that the beneficial increase of larval survival in the challenge test is due to cross-protection.

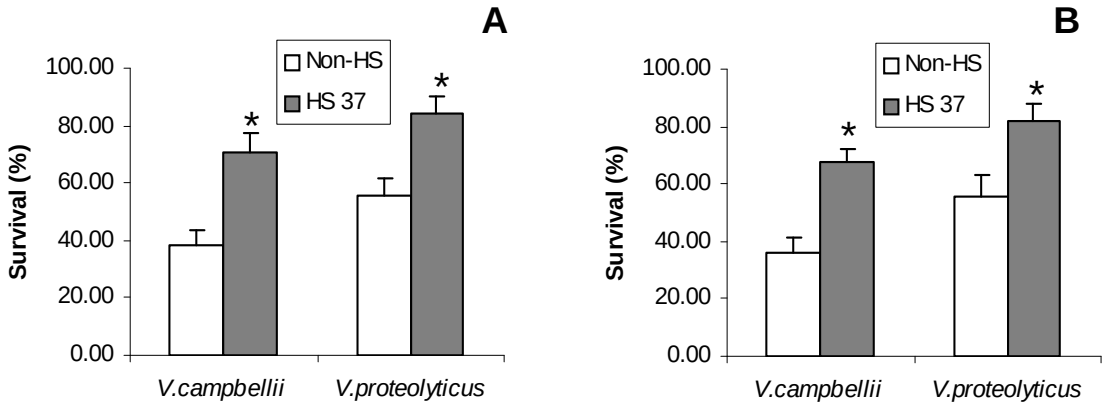

Fig. 4.1. Survival (%) after 24 h challenge test using 10^7 cells/ml of *Vibrio campbellii* and *Vibrio proteolyticus* at optimal HS condition (NLHS at 37°C for 30 min and 6 h recovery period). Each experiment was repeated twice: A and B. Asterisks (*) indicates significant difference (p<0.05) between HS treatment and control (Non-HS).

Hsp70 expression

Western blot detection of Hsp70 in the controls (non-HS) and heat shocked *Artemia* showed a cross-reaction with one protein band of approximately 70 kDa. There was a significant increase in the expression of Hsp70 in comparison to the control using equal amount of proteins (Fig. 4.2A, B) or equal amount of aliquots (results not shown).

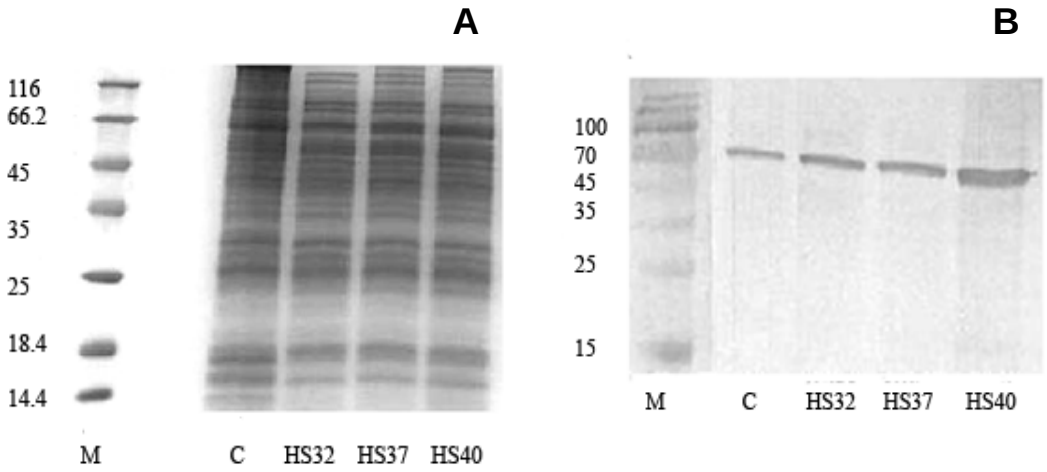

Fig. 4.2. Protein profiles of control (C) and heat-shocked (HS) treated *Artemia franciscana* larvae. (A) Approximately (50μg/μl) of larval protein was loaded in each lane of the gel and stained with Coomassie brilliant blue. (B) Alternatively, the gel was blotted and Western blot analysis was performed to detect members of the HSP-70 family. Protein standards (M) in kilodaltons are shown on the left of the figures.

Discussion

We have optimized the NLHS conditions as a way to protect *Artemia* nauplii against pathogenic bacteria. In this particular study on the effects of a non-lethal heat shock, gnotobiotically-grown *Artemia* nauplii were used throughout the experiment. The established gnotobiotic system was previously used in the evaluation of different yeast cell-wall mutants and microalgal strains as feed in *Artemia* (Marques et al., 2004a). With the same culture system, the immunostimulatory nature of β-glucans and baker´s yeast in a challenge test of *Artemia* was verified (Marques et al., 2006; Marques et al., 2004b). This gnotobiotic system provides an excellent and fully controllable host-pathogen environment. Furthermore it facilitates the investigation and verification of the effects of a NLHS towards a bacterial pathogen in *Artemia* nauplii. The experimental system avoids interference of factors such as modified physiology of host-associated microorganisms or even shift in microbial composition as the NLHS was applied in axenically hatched *Artemia* nauplii.

As often discussed, most physiological and environmental stressors may lead to impaired survival of cells or animals (Pedro et al., 1997; Braid et al., 2005; Xiao et al., 2005;

Varsamos et al., 2006). In addition, long-term stress increases the susceptibility to infectious diseases, for example in fish (Peters et al., 1988) and shrimps (Lee and Wickins, 1992). However, we observed a remarkable increase in the larval survival when a short-term stress was imposed on the challenged animals. HS-treated *Artemia* nauplii performed better in the *Vibrio* challenge test (*V. campbelli* and *V. proteolyticus*), while the controls (non-HS treated) were generally more susceptible to pathogenic vibrios. The optimal NLHS conditions consisted of temperature increases from 28°C to 37°C for 30 min, with subsequent recovery for 6 h at 28°C before exposure to the pathogen. Survival could be doubled in the *Vibrio* challenge test. An extended duration of the NLHS does not further increase the effects, while prolonged recovery periods showed a relative increase in mortality both in controls and treatments, which may be due to starvation as animals were not fed throughout the experiments. In agreement with the observation made by Marques et al., (2006a), it was noted that *V. campbellii* is slightly more virulent than *V. proteolyticus* as the mortality is higher in the challenge test.

Using Western blot analysis, we have furthermore documented (Miller and McLennan, 1988) that the application of a NLHS at optimal conditions increased the expression of the 70 kDa families of stress proteins. As previously documented, a 5 min HS at 40°C in 24 h larvae is sufficient to enhance the subsequent expression of p68 and p89 in nauplii. Two-dimensional IEF/SDS-PAGE analysis coupled to immunoblotting showed the p68 consist of inducible Hsp68 and Hsp70, and constitutively synthesized Hsc70 forms (Miller and McLennan, 1988). By one dimensional/SDS-PAGE and immunoblotting, we could detect the up-regulation of Hsp70 family. In adult *Artemia franciscana*, the low level of constitutive Hsc70 and Hsp67 was still strongly up-regulated by a sub-lethal HS at 37°C for 30 min (Frankenberg et al., 2000). Recent studies documented that isolated erythrocytes from the eurythermal species, *Fundulus heteroclitus* reached a maximum induction rate in the first

6 h of recovery (Koban et al., 1991) while maximal induction of Hsp70 were observed during a 6–9 h recovery in an endothermic cell line of the Chinese hamster ovary (CHO) cells culture (Lee et al., 1991), similar to the recovery period described by us, which in our case results in maximum protection against the pathogens.

Many studies continue to disclose the remarkable diversity and functions of Hsps in *Artemia*. Cross protection against a secondary insult were frequently observed after an initial stress. Concomitant with this stress, the expression of Hsps was observed. For instance, besides verifying that up-regulation of Hsp70 was associated with the protection of *Artemia* (nauplii and adult) against extreme thermal stresses (Clegg et al., 2000a; MacRae, 2003; Clegg et al., 2001; Robert, 2002), other Hsps such as p26 were also postulated to protect mammalian cells (Cos-1) against oxidative damage (Collins and Clegg, 2004) and further acts synergistically with trehalose to confer desiccation tolerance in mammalian cells (Ma et al., 2005). In this study, we verified if a primary stress by an application of a NLHS could protect *Artemia* nauplii against a subsequent pathogenic bacteria attack. Also in this case of gnotobiotic conditions, an up-regulation of the Hsp70 protein family by the applied NLHS was observed. Based on the current observations, the beneficial effects on survival can possibly be explained by an activation of the innate immune system of *Artemia* nauplii in the short term, while we anticipate, based on the many reports on this subject, that long-term stress for many animals might increase the vulnerability to a pathogenic attack.

Piles of information indicate that stress-regulated Hsps are capable of inducing strong immune responses in vertebrates (Robert, 2002, Pockley, 2003). Acting as a “red flag”, extracellular Hsps, as a result of necrosis, are postulated to alarm the immune system on the existence of a foreign invader and thereby activate a prompt and potent immune response (both innate and adaptive immunity) enabling the host to combat the disease (Stewart and Young, 2004). The lack of data and research on short-term stress and the immune response in

invertebrates, particularly in crustaceans, may have hampered the establishment of a clear association between Hsps production and activation of the innate immune response. Nevertheless, de la Vega et al. (2006) have recently demonstrated that a short-term hyperthermic treatment and the associated up-regulation of the Hsp70 protein family might play a substantial role in the beneficial reduction in the replication of gill-associated virus (GAV) in *Penaeus monodon*, postulating that Hsps play a vital role in the immune response of the whole organism.

The data reported in this paper do not provide evidence that the innate immune system in *Artemia* was triggered by Hsp70 expression. They merely illustrate that a NLHS, associated with a proven up-regulation of Hsp70 and probably other Hsps results in the protection against two pathogens. In view of the mounting evidence that Hsps can stimulate the innate immune response in vertebrates, we would like to put forward the hypothesis that Hsps are directly involved in triggering the innate immune response in the invertebrate *Artemia*. Unequivocal evidence for this hypothesis awaits the development of the proper genetic tools such as knock-out *Artemia* or the development of RNA interference (RNAi) technology (Copf et al., 2004) to add to the currently available gnotobiotic system.

Acknowledgements

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CHAPTER 5

Exposure of gnotobiotic *Artemia franciscana* larvae to abiotic stress promotes heat shock protein 70 and enhances resistance to pathogenic *Vibrio campbellii*

Yeong, Y. S., Pineda, C., MacRae, T. H.,

Sorgeloos, P. & Bossier, P.

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Abstract

Larvae of the brine shrimp *Artemia franciscana* serve as important feed in fish and shellfish larviculture, however they are subject to bacterial diseases that devastate entire populations and consequently hinder their use in aquaculture. Exposure to abiotic stress was shown previously to shield *Artemia* larvae against infection by pathogenic *Vibrio*, with the results suggesting a mechanistic role for heat shock protein 70 (Hsp70). In the current report combined hypothermic/hyperthermic shock followed by recovery at ambient temperature induced Hsp70 synthesis in *Artemia* larvae. Thermotolerance was also increased as was protection against infection by *Vibrio campbellii*, the latter indicated by reduced mortality and lower bacterial load in challenge tests. Resistance to *Vibrio* improved in the face of declining body mass as demonstrated by measurement of ash free dry weight. Hypothermic stress only and acute osmotic insult did not promote Hsp70 expression and thermotolerance in *Artemia* larvae, nor was resistance to *Vibrio* challenge augmented. The data support a causal link between Hsp70 accumulation induced by abiotic stress and enhanced resistance to infection by *V. campbellii*, perhaps via stimulation of the *Artemia* immune system. This possibility is now under investigation and the work may reveal fundamental properties of crustacean immunity. Additionally, the findings are important in aquaculture where development of procedures to prevent bacterial infection of feed stock such as *Artemia* larvae is a priority.

Introduction

Artemia are predominantly found in extreme habitats where few animals exist (Van Stappen, 2002) and the ability to tolerate environmental perturbations makes this aquatic crustacean an interesting model organism for stress response studies (Clegg and Trotman, 2002). For example, numerous studies address the effects of temperature and salinity, important physical factors in the life of this organism, on the survival of *Artemia* cysts, larvae and adults (Miller and McLennan, 1988a, b; Liang and MacRae, 1999; Frankenberg et al., 2000; Browne and Wanigasekera, 2000; Clegg et al., 2000a, b). Moreover, in these studies, including the pioneering work by Miller and McLennan (1988a, b) on p68 and p89, apparent equivalents to Hsp70 and Hsp90 respectively, several *Artemia* Hsps were identified. This work is important fundamentally because it is related to stress physiology and *Artemia* also have an essential role in aquaculture as a component of live diets, particularly in fish and shellfish larviculture (Sorgeloos et al., 1986).

Stress is a state where organismal homeostasis is either threatened or interrupted by intrinsic and/or extrinsic stimuli or stressors (Chrousos and Gold, 1992; Mercier et al., 2006). Aquatic organisms are regularly exposed to severe environmental and pathophysiological stresses (Song et al., 2006) that induce a cascade of molecular and physiological responses (Livingstone, 1985). Animals survive adverse conditions in several ways and one well characterized mechanism is by the induction of stress proteins, also termed heat shock proteins (Hsps). These proteins are synthesized constitutively in cells and they are induced after exposure to stress including heat, cold, oxygen deprivation, desiccation, infection and disease (Lindquist and Craig, 1988; Steinert and Pickwell, 1993; Parsell and Linquist, 1994; Jolly and Morimoto, 1999; Sørensen and Loeschcke, 2001). Besides cell maintenance during stress, Hsps assist in proper folding of proteins, prevention of protein aggregation and transport of proteins across membranes (Linquist, 1992). Moreover, Hsps play essential roles

in immune reactions of animals against infection and disease (Robert, 2003; Pockley, 2003), and cross-tolerance to environmental perturbations (DuBeau et al., 1998; Todgham et al., 2005).

Cross-tolerance or cross-protection is a mechanism whereby a primary stress transiently increases the resistance to other insults of the same or a different nature (Volker et al., 1992), thus allowing cells or animals to survive subsequent, more severe, stress (Jean et al., 2004). In line with these observations, induced Hsp synthesis confers secondary hyperthermic stress tolerance on *Artemia* (Clegg et al., 2000a; Frankenberg et al., 2000). In other work, a non-lethal heat shock promoted Hsp70 expression and cross-protected *Artemia* larvae against *Vibrio* challenge (Yeong et al., 2007). The present study extends this focus, featuring the differential expression of Hsp70 in *Artemia* larvae upon exposure to abiotic stressors. Additionally, the effects of these stresses on weight loss, induced thermotolerance, and immune response as revealed by survival after *Vibrio* challenge, were determined, revealing a potential relationship between Hsp70 and enhanced immunity in *Artemia* larvae.

Materials and Methods

Maintenance of bacteria

Vibrio campbellii (LMG 21363) isolates stored in 40% glycerol at –80°C were grown at 28°C on marine agar. Marine broth 2216 (Difco Laboratories, Detroit, MI) was then inoculated with single colonies and incubated overnight with constant shaking at 28°C to stationary phase. Bacterial cells were harvested under aseptic conditions by centrifugation at 2200 x *g* for 15 min prior to suspension in sea water which had been filtered and autoclaved, as used throughout the study. Culture densities were determined spectrophotometrically at 550 nm and bacterial numbers were calculated according to the McFarland standard

(BioMerieux, Marcy L'Etoile, France), where an optical density of 1.0 corresponds to 1.2 x 10^9 cells/ml.

Preparation of axenic *Artemia* larvae

Axenic *Artemia* larvae were obtained essentially as described (Marques et al 2004a). One g of high-hatching *A. franciscana* cysts from the Great Salt Lake, Utah, USA (EG® Type, INVE Aquaculture, Belgium) was hydrated in 90 ml of tap water for 1 h with vigorous aeration and then transferred to a laminar flow hood for decapsulation. Fifty ml of cold sodium hypochlorite containing 15% (w/v) active chlorine and 3.3 ml of 32% (w/v) sodium hydroxide were added to the hydrated cysts, followed after 150 s by 70 ml of autoclaved sodium thiosulphate pentahydrate at 10 mg/l. Decapsulated cysts were washed several times with sea water and collected over 50 μm sterile sieves. A few mg of cysts were transferred to separate, sterile 50 ml Falcon tubes containing 30 ml of sea water and capped. The tubes were incubated at 28°C for 18-24 h with rotation at 4 cycles per min and constantly exposed to incandescent light at ± 41 μEm^{-2}. Subsequently, hatched larvae at stage 2 of development, when the mouth has opened and bacterial ingestion can occur, were harvested within the next 4-6 h.

Thermal and osmotic stress of *Artemia* larvae

Axenically hatched larvae acclimated at 28°C were transferred to sea water at 4°C in sterile 500 ml bottles and held for 1 h at 4°C with aeration prior to incubation at 28°C for 6 h, a treatment termed CS1. Larvae incubated at 4°C were also exposed to a non-lethal heat shock of 37°C (Δt = 5°C min^{-1}) for 30 min, followed by recovery for 6 h at 28°C, a procedure termed CS2. Air used during culturing was passed through a 0.22 μm filter.

Axenically hatched *Artemia* larvae, held normally at a salinity of 30 g/l, were exposed for 30 min to osmotic stress including a hypotonic shock at 4 g/l and hypertonic shocks at 50, 100 and 150 g/l. For osmotic stress under axenic conditions, larvae acclimated at 30 g/l were collected on cloth filters and transferred to 500 ml aerated bottles containing 50 ml of salt solutions at 4, 50, 100 and 150 g/l. Salt solutions consisted of Instant Ocean® synthetic salt, Aquarium Systems Inc., France, sterilized by autoclaving and diluted with autoclaved distilled water. After stress, larvae were acclimated to normal salinity for 6 h, either by adding salt to the hypotonic solution or by diluting the hypertonic solutions with distilled water. Larvae were then harvested and used for protein characterization, ash free dry weight measurements, induced thermotolerance experiments and *V. campbellii* challenge tests.

Protein extraction, SDS polyacrylamide gel electrophoresis and immunoprobing of western blots

Protein extraction was performed essentially as described previously (Clegg et al., 2000a; Yeong et al., 2007). *Artemia* larvae were collected on 50 μm sieves and rinsed with ice-cold distilled water. Two hundred mg ml^{-1} (wet weight) of tissue was homogenized in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) (Clegg et al., 2000a), and supplemented with protease inhibitor cocktail (Catalogue # P8340, Sigma-Aldrich, Inc. USA) as recommended by the manufacturer. Aliquots of homogenate were combined with equal volumes of SDS sample buffer, vortexed, heated at 95°C for 5 min (Laemmli, 1970), cooled and centrifuged at 2200 x *g* for 1 min.

Ten μl samples representing 1.0 mg (wet weight) of animals were applied to each lane of a 10% SDS polyacrylamide gel (Frankenberg et al., 2000; Clegg et al 2000a) prior to electrophoresis. Two gels were run simultaneously and one was stained with Coomassie

Biosafe (BioRad Laboratories, USA). Proteins in the second gel were transferred to a polyvinylidene fluoride transfer membrane (BioRad Immun-Blot^{TM} PVDF, USA) for antibody probing. Membranes were incubated in blocking buffer (50 ml of phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin) for 60 min at 25°C. Mouse monoclonal anti-Hsp70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO), which recognizes both constitutive and inducible Hsp70 (Yeong et al., 2007), was used as primary antibody at the recommended dilution of 1:5000. Donkey anti-mouse IgG coupled with horseradish peroxidase conjugate (HRP) (Affinity BioReagents Inc., Golden, CO) was employed as secondary antibody at the recommended dilution of 1:2500. Detection was performed with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate as substrate in association with 0.01% (v/v) H_2O_2 in 0.1 M Tris-HCl (pH 7.6).

Determination of larval ash free dry weight

To determine ash free dry weight *Artemia* larvae were collected over a 50 μ cloth filter and washed several times with autoclaved distilled water. One hundred larvae from each treatment were placed in triplicate in separate porcelain cups cleaned previously with formic acids, dried and weighed. The samples were heated 4 h at 103°C for dry weight measurement, then combusted at 600°C for 6 h to determine ash content. The ash free dry weight was calculated as the difference between the total dry weight and the ash weight.

Determination of induced thermotolerance in *Artemia* larvae

After recovery from an initial stress, 50 larvae were transferred in triplicate into separate Falcon tubes containing 30 ml of sea water, exposed to heat shock at 42°C for 30 min (Clegg et al., 2000a) and then transferred to 28°C. Induced thermotolerance was

determined 12 h after heating by counting actively swimming larvae. The experiments were repeated once.

V. campbellii challenge tests with *Artemia* larvae

For bacterial challenge, 50 larvae were incubated at 28°C in each of six sterile Falcon tubes containing 30 ml of sea water. Three tubes received bacteria and three did not, the latter used for assessing bacterial contamination by incubating 100 µl of culture medium on marine agar 2216 (Marques et al., 2004a) for 5 days at 28°C. If contamination was detected the experimental results were discarded. Challenge tests were performed as described (Marques et al., 2006a) except *V. campbellii* (LMG 21363), a relatively virulent pathogen for gnotobiotically-grown *Artemia* larvae (Marques et al., 2006a, b, c; Defoirdt et al., 2006a) was added to 1 x 10^7 cells/ml. Survival was determined 24 and 36 h after challenge by collecting actively swimming larvae and fixing in Lugol's solution prior to counting. Survival percentage was calculated as N_t x 100 / N_o where N_t and N_o are final and initial numbers of larvae, respectively.

Colonization of *Artemia* larvae by *V. campbellii*

Artemia larvae subjected to treatments CS1 and CS2 were harvested 8 h after *Vibrio* challenge by sieving on sterile 150 µm pore size nylon filters and then rinsed twice with 10 ml of autoclaved nine-salts solution (NSS) (17.6 g/l NaCl, 1.47 g/l Na_2SO_4, 0.08 g/l $NaHCO_3$, 0.25 g/l KCl, 0.04 g/l KBr, 1.87 g/l $MgCl_2$, 0.41 g/l $CaCl_2$, 0.008 g/l $SrCl_2$ and 0.008 g/l H_3BO_3). Ten larvae, transferred to sterile plastic bags containing 10 ml of NSS, were homogenized with a stomacher blender (400SN, Seward Medical, UK) for 10 min. The homogenates were transferred to Falcon tubes, 10-fold serial dilutions were prepared, and samples were plated on marine agar with a Spiral-plater (Spiral Systems Inc., USA) prior to

incubation at 28°C for 24 h and colony counting. The experiments were conducted in duplicate with each test performed in triplicate.

Statistical analyses

Values for larval survival (%) were ArcSin to satisfy normality and homocedasticity requirements whenever necessary. For *V. campbellii* load, the CFU values were log-transformed. Significant differences in larval survival and *V. campbellii* colonization were determined by performing one-way ANOVA followed by Tukey test at a significance level of 0.05. All statistical analyses were performed with software SPSS® version 11.5 for Windows®.

Results

Synthesis of Hsp70 in stressed *Artemia* larvae

Coomassie staining of SDS polyacrylamide gels clearly demonstrated increased amounts of a 70 kDa polypeptide in CS2 samples (Fig 5.1A). Immunoprobing of western blots with a monoclonal antibody to Hsp70 revealed a single co-migrating polypeptide of approximately 70 kDa, which as shown in stained gels, increased only in CS2 samples (Fig 5.1B). In contrast, CS1 treatment yielded a minor reduction in the antibody-reactive 70 kDa protein, and the protein was indifferent to osmotic stress. The same results were obtained in two independent experiments for which figure 5.1 is a representative example.

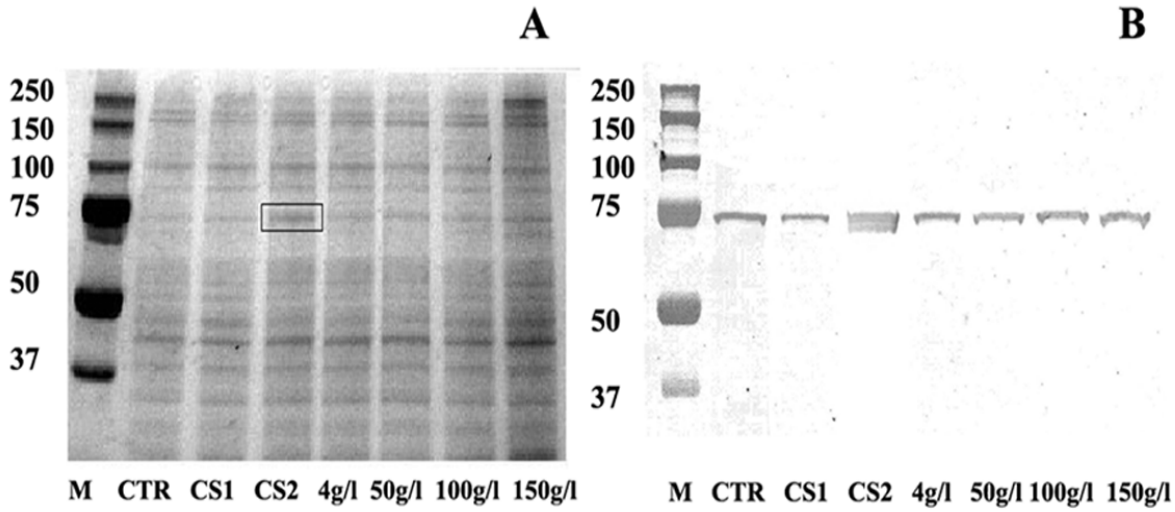

Fig 5.1. (A) SDS polyacrylamide gel electrophoresis of protein samples from stressed *Artemia* larvae. Ten µl samples containing equivalent amounts of protein were loaded in each lane. (B) Immunodetection of Hsp70 on western blots; CTR, non-stressed larvae; CS1, cold shock at 4°C for 1 h followed by recovery at 28°C for 6 h; CS2, cold shock at 4°C followed by heat shock at 37°C for 30 min and recovery at 28°C for 6 h; 4 g/l, hypotonically-stressed larvae exposed to a 4 g/l salt solution for 30 min followed by recovery for 6 h; 50 g/l, 100 g/l and 150 g/l, hypertonically-stressed larvae exposed to 50, 100 and 150 g/l salt solution for 30 min followed by recovery for 6 h; M, protein standards in kDa. Box, 70 kDa protein.

Larval ash free dry weight is stress dependent

Animals osmotically stressed at 100 and 150 g/l and those from the CS2 treatment had lower ash free dry weights than non-stressed larvae (Fig 5.2). In contrast, no significant differences were detected in ash free dry weight of larvae subjected to CS1 treatments and osmotic stresses at 4 and 50 g/l ($p>0.05$).

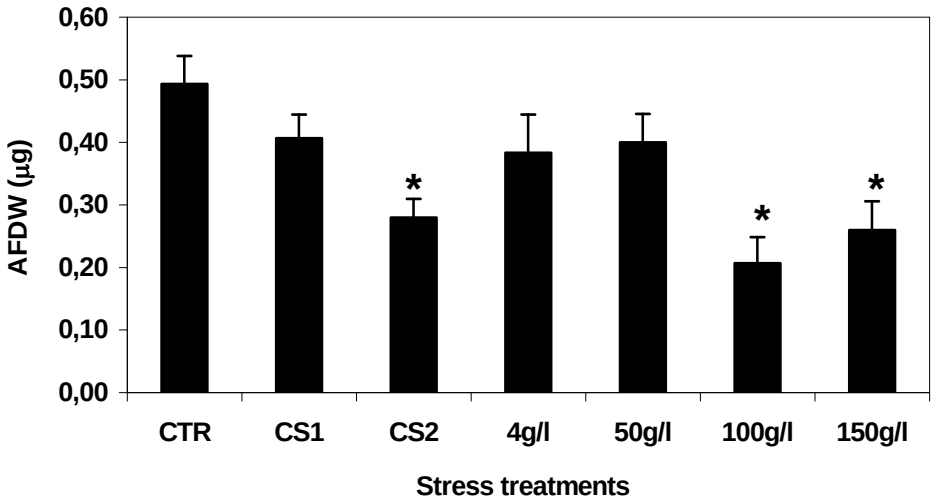

Fig 5.2. Ash free dry weight of stressed *Artemia* larvae. One hundred larvae were heated 4 h at 103°C for dry weight measurement, then combusted at 600°C for 6 h to determine ash content. The ash free dry weight was calculated as the difference between the total dry weight and the ash weight. Refer to Fig. 5.1 for explanation of sample designations. * indicates a significant difference (p<0.05) between the stress treatment and control.

Induced thermotolerance in larvae occurred only in response to CS2 treatment

Thermotolerance in *Artemia* larvae exposed to the CS2 treatment was enhanced whereas larvae subjected to CS1 treatment and osmotic stress were less resistant to temperature increase (Fig 5.3).

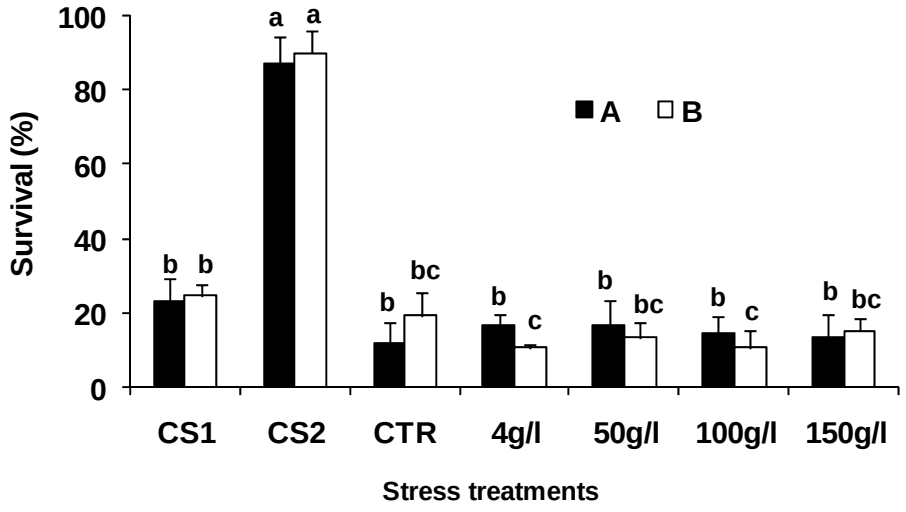

Fig 5.3. Thermotolerance induction in *Artemia* larvae. Non-stressed (CTR) and stressed *Artemia* larvae were exposed to heat shock at 42°C for 30 min. Induced thermal tolerance was based on larval survival after 12 h of heat shock. Experiments were repeated once with replicates indicated by A and B. Refer to Fig. 5.1 for explanation of sample designations. Values showing the same superscript letter for each experiment are not significantly different ($p>0.05$).

Enhanced survival of CS2 stressed larvae in *V. campbellii* challenge tests

In replicate experiments, approximately 80% of non-stressed and unchallenged larvae survived incubation at 28°C (Fig 5.4) whereas about 40% of non-stressed larvae challenged with *V. campbellii* were viable. CS2 treated larvae exhibited substantially higher survival as compared to non-stressed challenged controls while a small but significantly reduced survival ($p<0.05$) occurred for larvae experiencing CS1 treatment. Larvae osmotically-stressed at 4, 100 and 150 g/l salinity had significantly lower survival ($p<0.05$) than non-stressed animals upon exposure to *V. campbellii* and for the latter viability was very low (Fig 5.5). Approximately 90% and 72% of the unchallenged larvae were alive at 24 and 36 h respectively in these experiments (not shown).

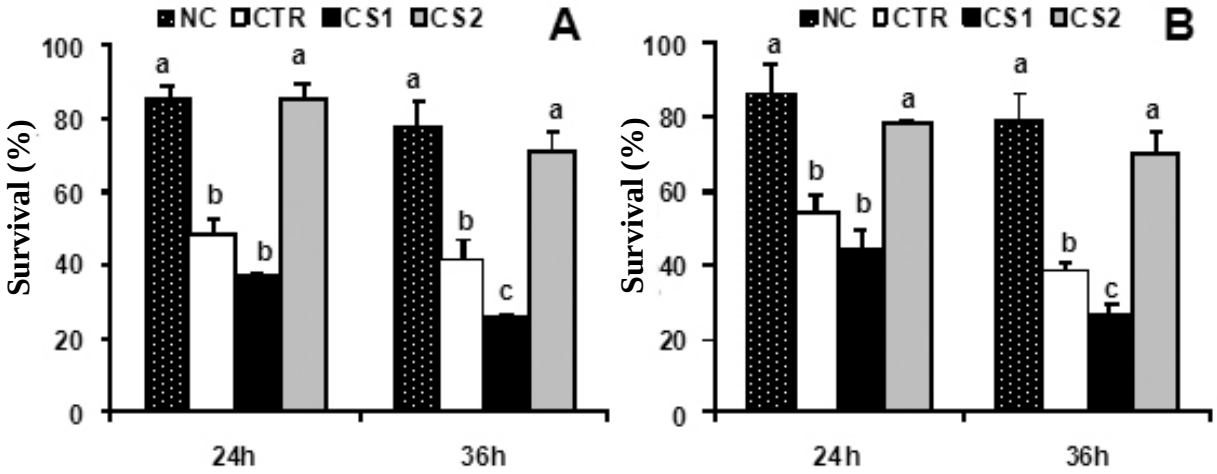

Fig 5.4. Survival of *Artemia* larvae in *V. campbellii* challenge tests. After CS1 and CS2 treatments larvae were exposed to a *V. campbellii* challenge of 1 x 10^7 cells/ml. Live larvae were counted 24 h and 36 h after challenge and each experiment was repeated once, with replicates labelled A and B. NC, non-stressed and unchallenged larvae; CTR, non-stressed larvae with *V. campbellii* challenge; CS1 and CS2, refer to Fig. 5.1 for explanation. Values in matching time columns with the same superscript letter are not significantly different ($p>0.05$).

Reduced *V. campbellii* load in CS2 stressed larvae

Approximately 8.7 x 10^3 *V. campbellii* accumulated per non-stressed larva (Fig 5.6) with higher numbers generally present after CS1 treatment, although differences were not significant. In contrast, reductions of approximately 49% and 61% occurred in the number of *V. campbellii* per larva after CS2 treatments ($p<0.05$).

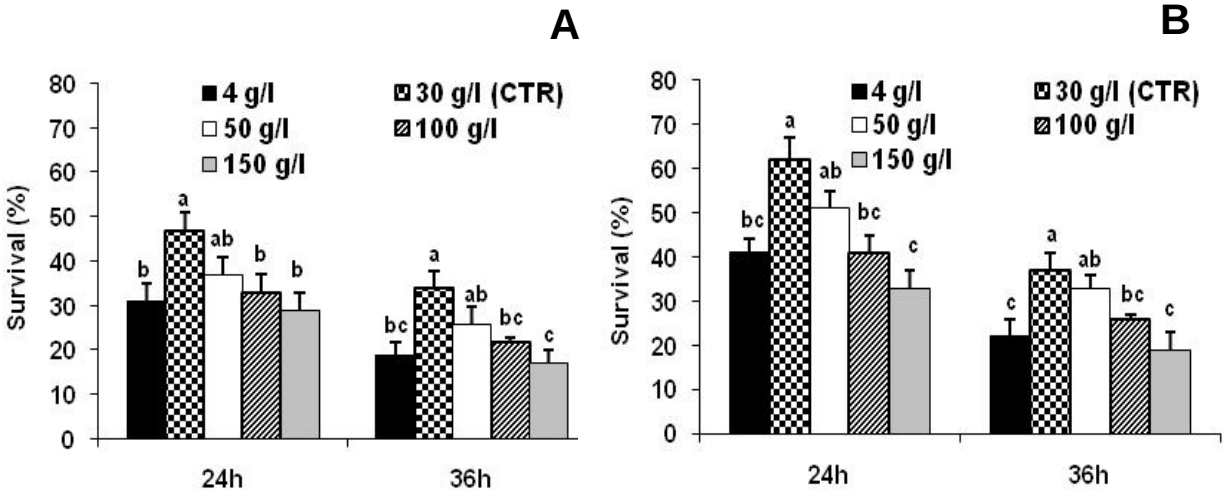

Fig 5.5. Survival of osmotically stressed *Artemia* larvae after challenge tests with *V. campbellii*. Live larvae were counted 24 h and 36 h after challenge with 1 x 10^7 *V. campbellii* per ml. Each experiment was repeated once and replicates are labelled A and B. Refer to Fig. 5.1 for explanation of sample designations. Values in the same column for each experiment with matching superscript letters are not significantly different (*p>0.05*).

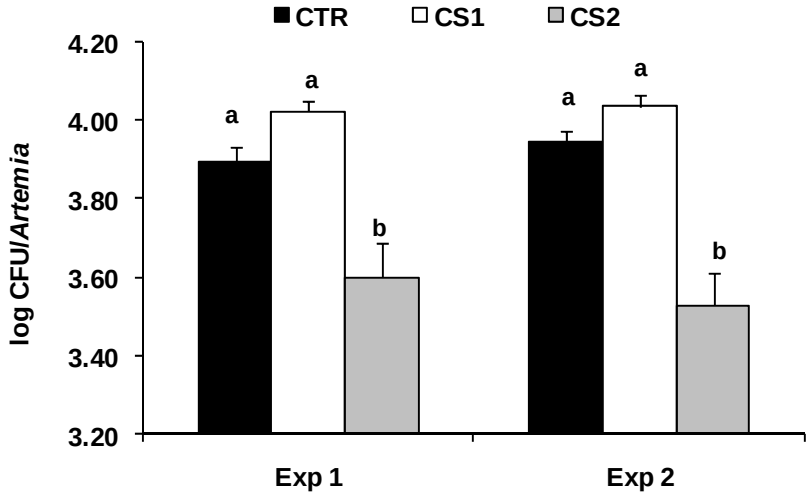

Fig 5.6. *Vibrio* colonization of *Artemia* larvae. Larvae from CS1 and CS2 treatments were exposed to 1 x 10^7 *V. campbellii* per ml for 8 h prior to collection of 10 animals and counting of bacteria. The experiment was repeated once with replicates labelled Exp 1 and Exp 2. CTR, non-stressed larvae with *V. campbellii* challenge; CS1 and CS2; refer to Fig. 5.1 for explanation of sample designations. Values in the same column for each experiment with matching superscript letters are not significantly different (*p>0.05*).

Discussion

Exposing gnotobiotic *Artemia* larvae to 4°C for 1 h followed by an abrupt non-lethal heat shock at 37°C for 30 min and a 6 h recovery is shown in this report to induce Hsp70 production. Conversely, a small reduction in the amount of Hsp70 was observed in larvae cooled from 28°C to 4°C and then warmed to 28°C. Stress often stimulates Hsp expression in aquatic organisms with Hsp70 induced in hypoxic Nile tilapia *Oreochromis niloticus (L.)* juveniles (Delany and Klesius, 2004), Indian major carp, *Cirrhinus mrigala* (Ham.) (Das et al., 2005) and the tiger prawn, *P. monodon* (de la Vega et al., 2006). A temperature increase from 21 to 37°C for 30 min followed by a 24 h recovery strongly induced Hsp70 in *Artemia* adults (Clegg et al., 2000a) and a non-lethal heat shock from 28 to 37°C for 30 min with a 6 h recovery triggered Hsp70 production in gnotobiotic-grown larvae (Yeong et al., 2007). In contrast to the situation with thermal perturbation, neither hypotonic nor hypertonic osmotic stress for 30 min induced Hsp70 production in *Artemia* larvae. Likewise, the absence of Hsp70 induction was observed in hypo-osmotic stressed *P. monodon* obtained by switching from 35 to 10g/l for 8 h (de la Vega et al., 2006). Although *Artemia* normally adapt readily to changing salt concentrations (Browne and Wanigasekera, 2000), larvae exposed to salinities of 100 and 150g/l for 30 min experienced significant weight loss. This may have resulted from reduced energy reserves as a consequence of increased metabolic activity required to cope with the imposed stress, a result seen in rats (Harris et al., 1998).

Stress may compromise organismal immune response and increase vulnerability to infection. As an example, temperature stress increases the susceptibility of sea bass, *Dicentrarchus labrax* to nodavirus (Varsamos et al., 2006). Additionally, thermal stress reduces resistance of the tiger shrimp *P. monodon* to *P. damselae subsp. damselae* (Wang and Chen, 2006b), and the White shrimp *L. vannamei* is more prone to *V. alginolyticus* infection after heating (Cheng et al., 2005). Transfer of *P. monodon* from 25 to 5 ppt and

from 15 to 35 ppt salinity reduced immune capability and decreased resistance against *P. damselae subsp. damselae* infection (Wang and Chen, 2006a). H_2O_2 accelerates the mortality of *Tenacibaculum maritimum* infected turbot (Avendaño-Herrera et al., 2006), while metal stress influences disease transmission and susceptibility of aquaculture species (Liao et al., 2006). Furthermore, hypoxic-stressed *Penaeus styliriostris* are more sensitive to infection by *V. alginolyticus* (Le Moullac et al., 1998). Collectively, these studies indicate that stress suppresses immunity, leading to increased vulnerability to infections and greater mortalities. In agreement with these data, gnotobiotic *Artemia* larvae experiencing CS1 treatment and exposure to high salt were less resistant to *V. campbellii* than were non-stressed animals.

Although many stresses reduce immunity, a non-lethal heat shock may cross-protect against further insult, a phenomenon usually correlated with Hsp production and defence against subsequent environmental disturbance. Thermal shock of salmon guards against a subsequent severe osmotic challenge, perhaps due to Hsp70 induction (DuBeau et al., 1998), and the induced expression of Hsps in most fishes by high temperature is correlated with increased resistance to a second heat stress (Basu et al., 2002). A sub-lethal heat shock promotes Hsp70 accumulation in adult *Artemia* and shields against exposure to lethal heat shock (Frankenberg et al., 2000). In this study, stressed gnotobiotic *Artemia* larvae undergoing CS2 treatment produced Hsp70, indicating this protein is directly involved in conferring enhanced heat tolerance. Furthermore, combined hypothermic and hyperthermic stress up-regulates Hsp70, and this is associated with cross-protection against *V. campbellii*. Unexpectedly, these animals exhibited a significantly lower ash free dry weight even though a two-fold increase in larval survival was recorded after *V. campbellii* challenge. Additionally, stressed larvae failing to accumulate Hsp70 lacked protection against *V. campbellii*, agreeing with earlier results indicating a role for Hsp70 in cross-protection of *Artemia* larvae (Yeong et al., 2007).

On an applied note, it is common practice in commercial aquaculture to store *Artemia* larvae in the cold thus reducing the number of daily harvests, preventing larvae from molting, prolonging storage, and preserving biomass, all with economic advantage (Mercie, 1996). The transfer of cold-stored larvae to fish tanks often involves sudden temperature increases because 28°C is an optimal rearing condition for most warm water aquaculture species. Our results suggest that such larvae have increased sensitivity to opportunistic pathogens like *Vibrio* species.

How Hsp70 and other Hsps protect against pathogenic *V. campbellii* is unclear, but extracellular Hsps are known to regulate the innate immune response (Pockley, 2003; Chen et al., 1999). For instance, the heat-induced synthesis of small heat shock proteins and Hsp90 triggers *C. elegans* immunity to pathogenic *Pseudomonas aeruginosa* (Singh and Aballay, 2006a). The mechanism may involve heat shock transcription factor-1 and the associated DAF2/DAF-16 pathway which regulates aging and immunity in nematodes (Singh and Aballay, 2006b). Furthermore, extracellular Hsp72 robustly promotes inflammatory cytokine production (Johnson and Fleshner, 2006) and may stimulate production of inducible nitric oxide synthase (Panjwani et al., 2002; Campisi and Fleshner, 2003), tumour necrosis factor α , interleukin-1β and IL-6 (Asea et al., 2000; Campisi and Fleshner, 2003), all known to modulate infection. Substantial evidence indicates that Toll-like receptors 2 and/or 4, which act as cell surface receptors for extracellular Hsp72, transduce inflammatory signals to innate immune cells such as macrophages, dendritic cells and neutrophils (Visintin et al., 2001; Vabulas et al., 2002; Asea et al., 2002; Ménoret, 2004). Findings presented in this report support the emerging idea that Hsps activate innate immune responses in *Artemia* and other invertebrates, thereby protecting against pathogens such as *V. campbellii*. These observations are of fundamental importance in understanding invertebrate immune function and they have significant potential for application in aquaculture.

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CHAPTER 6

DnaK, the 70-kDa bacterial heat shock protein, protects *Artemia* larvae from *Vibrio* infection

Yeong, Y. S., Ashame, M. F., Chen, S. J., MacRae, T. H.,

Sorgeloos, P. & Bossier, P.

(Submitted)

Abstract

Heat shock proteins are potent immunomodulators, a characteristic reflecting their potential as therapeutic agents and which led to their application in combating infection. As an example, the up-regulation of endogenous Hsp70 in the branchiopod crustacean *Artemia franciscana* is concurrent with shielding against bacterial infection. In experiments described herein to better understand this protective mechanism, feeding gnotobiotic *Artemia* with *Escherichia coli* over-producing different prokaryotic Hsps increased larval resistance to *Vibrio campbellii*. Immunoprobing of western blots showed that enhanced resistance to *V. campbellii* correlated with DnaK production in *E. coli*. A definitive role for DnaK was then demonstrated by feeding *Artemia* larvae with transformed bacteria over-producing only this protein, although other Hsps such as DnaJ and grpE also provided tolerance against *Vibrio* infection. Interestingly, stress-induced up-regulation of endogenous Hsp70 was shown previously to associate with increased survival of *Artemia* larvae upon *Vibrio* challenge. Protection may be mediated via an immunostimulatory mechanism and this is under investigation. Whatever the outcome of these studies, the current investigation indicates that feeding of bacteria synthesizing selected Hsps is an alternative to antibiotic use as a way to boost the resistance of *Artemia* larvae to bacterial infection, a finding with potential applications in aquaculture.

Introduction

Bacterial cells, either dead or alive, are used as alternatives to antibiotics in aquaculture to control disease by enhancing the resistance of larvae to pathogenic infection (Gullian et al., 2004; Panigrahi et al., 2005; Taoka et al., 2006; Salinas et al., 2005; 2006; Diaz-Rosales et al., 2006). Improved survival may reflect stimulation of the immune system, leading to more effective phagocytosis of harmful microorganisms by macrophages along with enhanced production of antibodies and interferon (Farzanfar, 2006). Moreover, ingested bacteria serve as nutrients for larvae, contributing directly to their growth and well being (Hessen and Anderson, 1990; Orozco-Medina et al., 2002; Martínez-Díaz et al., 2003; Rengpipat et al., 2003; Rombaut et al., 2003; Villamil et al., 2003; Planas et al., 2004; Venkat et al., 2004; Vine et al., 2004; Macey and Coyne, 2005). Beneficial bacteria, known as probionts, exist and these include lactic acid producing strains and *Bacillus* spp. Probionts control host colonization and proliferation of pathogenic bacteria presumably by producing antagonistic compounds such as acids, hydrogen peroxide and bacteriocins (Farzanfar, 2006). Probionts improve the nutritional value of feed by forming a balanced flora in the host gut, which contributes digestive enzymes (Reid, 1999; Verschuere et al., 2000a; Vázquez et al., 2005).

The brine shrimp *Artemia* is used widely as live feed in larviculture (Sorgeloos et al., 2001), and as an excellent model organism for studying disease in penaeid shrimp, lobsters and other crustaceans (Overton and Bland, 1981; Criado-Fornelio et al., 1989; Verschuere et al., 1999, 2000b; Yeong et al., 2007). The establishment of gnotobiotic *Artemia* test systems (Marques et al., 2004a), in which animals are cultured with known microbial populations, has facilitated mechanistic studies of host-microbe interactions and proved instrumental in developing bio-control strategies for aquaculture (Marques et al., 2004a, b, 2005, 2006; Defoirdt et al., 2006, 2007; Soltanian et al., 2007). There is now compelling evidence that

endogenous heat shock proteins (Hsps) influence the immune response of *Artemia franciscana*, contributing to tolerance of *V. campbellii* and *V. proteolyticus* (Yeong et al., 2007). These observations are extended in this report by determining the protective properties of exogenous Hsps. Specifically, *E. coli* synthesizing different Hsps were fed to *Artemia* larvae under gnotobiotic conditions demonstrating that DnaK and perhaps other Hsps increase the ability of *Artemia* larvae to survive *Vibrio* challenge.

Materials and Methods

Bacteria strains and plasmids

E. coli K12 strains CAG 626 and CAG 629, described in the NIH Recombinant DNA Advisory Committee (RAC) guidelines (Federal Register, 1986) as non-pathogenic bacteria, are similar except that CAG 629 does not express Hsps. Both strains were stored in 20% glycerol at –80°C and grown at 28°C for 48 h in LB-medium prior to heat shock. Recombinant plasmids pG-KJE8, pGro7 and pKJE7 (Chaperone Plasmid Set, TaKaRa© Bio. INC, Japan) respectively containing cDNAs encoding DnaK-DnaJ-grpE plus GroES-GroEL, GroES-GroEL, and DnaK-DnaJ-grpE, were transformed separately into *E. coli* CAG 626 (Cohen et al., 1972), yielding clones P1, P2 and P3. Empty plasmid pACYC184, obtained from *E. coli* K-12 strain K514 (λ) (BCCB™/LMBP, Belgium) by the Miniprep protocol (Sambrook et. al., 1989), was transformed into *E. coli* CAG 626, yielding strain P0.

DnaK cDNA from plasmid pKJE7 was amplified by PCR and cloned into the TOPO® cloning vector using a pBAD Directional TOPO® Expression Kit (Invitrogen™, Carlsbad, CA) as recommended by the manufacturer. Reactions were performed in a 50 µl PCR Master Mix (Promega, USA) containing 1 unit of proofreading polymerase (Platinum® *Taq* DNA polymerase, Invitrogen, Carlsbad, CA) and 0.2 µM each of oligonucleotide primers $HSP70_{forward}$ 5′-CACCATGGGTAAAATAATTGGT-3′ and $HSP70_{reverse}$ 5′-

TTATTTTTTGTCTTTGAC-3′. Denaturation at 95°C for 5 min was followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 1 min followed by 10 min at 72°C. cDNA fragments were ligated into the TOPO cloning vector and transformed into One shot TOP10 cells which were grown on LB agar containing 100 μg/ml ampicillin at 37°C. A bacterial clone containing DnaK cDNA was isolated from the LB plates and labelled YS2. Strain YS1 which does not express DnaK upon induction contained a short DNA fragment obtained by annealing two complementary primers, blHSP70$_{forward}$ 5′-CACCATGTAATGATGCCC-3′ and blHSP70$_{reverse}$ 5′-GGGCATCATTACATGGTG-3, and ligating into the TOPO® cloning vector. Two in frame stop codons prohibited the production of protein. Recombinant plasmids were then transformed into TOP10 cells. The characteristics of transformed bacterial strains and the plasmids used in this study are summarized in Table 6.1. *Vibrio campbellii* strain (LMG 21363) was employed as a pathogen for *Artemia* larvae (Marques et al., 2005; Yeong et al., 2007).

Induction of bacterial Hsp synthesis

Hsp synthesis was induced by heating *E. coli* CAG 626 and CAG 629, initially incubated at 28°C for 48 h, to 37°C at a Δt of 4°C/min and then maintaining this temperature for 30 min. In the *E. coli* strains P0, P1, P2 and P3, Hsps were induced by adding L-arabinose and/or tetracycline as recommended by the manufacturer. Maximum expression of DnaK in YS2 cells was determined to occur with 0.5 mg/ml L-arabinose for 4 h. After induction, bacteria were harvested by centrifugation at 2200 x *g* for 15 min at 38°C. Pellets were washed once with sea water and then suspended in 30 ml of the same medium. Sea water was autoclaved and filtered. Bacteria were quantified spectrophotometrically at 550 nm and fed immediately to *Artemia* larvae.

Table 6.1 - Transformed bacterial strains and plasmids

Bacteria strains	Plasmids	Induction	Hsps encoded by plasmids
PO	pACYC184 (BCCB™/LMBP Belgium)	L-arabinose (0.5 mg/ml for 1h)	-
P1	pG-KJE8 (TaKaRa© Bio. INC. Japan)	L-arabinose (0.5 mg/ml for 1 h) Tetracyclin (5 ng/ml for 1 h)	DnaK-DnaJ-grpE GroES-GroEL
P2	pGro7 (TaKaRa© Bio. INC. Japan)	L-arabinose (0.5 mg/ml for 1 h)	GroES-GroEL
P3	pKJE7 (TaKaRa© Bio. INC. Japan)	L-arabinose (0.5 mg/ml for 1 h)	DnaK-DnaJ-grpE
YS1	pblDnaK (constructed using pBAD TOPO® vectors, Invitrogen™, Carlsbad, CA)	L-arabinose (0.5 mg/ml for 1 h)	-
YS2	pDnaK (constructed using pBAD TOPO® vectors, Invitrogen™, Carlsbad, CA)	L-arabinose (0.5 mg/ml for 4 h)	DnaK

E. coli strain CAG 626 was transformed to yield strains P0-P3 whereas *E. coli* One Shot® TOP10 was transformed to yield YS1 and YS2.

Bacteria as feed for axenic *Artemia*

Axenic *Artemia* larvae were obtained by decapsulation and hatching (Sorgeloos et al., 2001; Yeong et al., 2007). Axenity was tested by incubating 100 µl of medium from *Artemia* cultures in marine agar for 5 days at 28°C and if contamination was detected experimental results were discarded. To examine the nutritional value of induced and non-induced bacteria, axenically cultured *Artemia* larvae were fed once with approximately 1 x 10^7 cells/ml of each bacterial strain except *V. campbellii*. Swimming larvae were collected after two days, fixed in Lugol's solution and counted. Survival percentage was calculated as N_t x 100 / N_o where N_t and N_o are final and initial numbers of larvae, respectively. Individual length was ascertained

by measuring fixed larvae with a dissecting microscope equipped with a drawing mirror, a digital plan measure and the software Artemia 1.0® (courtesy of Marnix Van Domme). Larval numbers were ArcSin to satisfy normality and homocedasticity requirements while individual length was either logarithmic or square root transformed as necessary. Significant differences in larval numbers and individual lengths were determined by analysis of variances (ANOVA) and Tukey's multiple comparisons range using statistical analysis software SPSS® version 11.5 for Windows®. Experiments for testing the nutritional value of bacterial strains and resistance to *Vibrio* challenge, as described in the following section, were performed twice with each replicate done in triplicate.

Protection of *Artemia* larvae against *V. campbellii*

Approximately 1 x 10^7 cells/ml of bacterial strains P0, P1, P2 and P3, either induced or non-induced, were incubated individually with axenic *Artemia* larvae for 6 h. Challenge tests were then performed as described (Marques et al., 2006) except approximately 1 x 10^7 *V. campbellii*/ml were added and incubation was with constant agitation and illumination. Survival was determined after 36 h by counting live *Artemia* larvae as described above and individual lengths were measured unless survival was less then 20%. To further examine bacterial strain P3, which in the previous experiment protected *Artemia* larvae most effectively against *Vibrio* challenge, Hsps were induced with L-arabinose at 0, 0.5, 1.0, 2.0 and 4.0 mg/ml for 1 h. Subsequently, L-arabinose at 0.5 mg/ml, which gave the best induction in initial experiments, was tested at 0, 7.5, 15, 30 and 60 min (Nishihara et al., 1998, 2000).

Artemia larvae were incubated for 6 h with YS1 and YS2 at 1 x 10^7 cells/ml to test their ability to protect *Artemia* larvae against *Vibrio* infection. Challenge tests were as described except larval survival was determined after 24 h.

Protein extraction, SDS polyacrylamide gel electrophoresis and immunoprobing of western blots

Bacteria were homogenized by rapid agitation with 0.1 mm diameter glass beads in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) containing protease inhibitor cocktail (Catalogue # P8465, Sigma-Aldrich, Inc. USA) at the highest recommended level. Subsequent to centrifugation at 2200 x *g* for 1 min at 4^{o}C, supernatant protein concentrations were determined by the Bradford method. Supernatant samples were then combined with equal volumes of 2 x SDS polyacrylamide gel buffer, vortexed, heated at 95°C for 5 min and electrophoresed in 10% SDS polyacrylamide gels (Laemmli, 1970), with each lane receiving equivalent amounts of protein. Gels were either stained with Coomassie Biosafe (BioRad Laboratories, USA) or transferred to polyvinylidene fluoride membranes (BioRad Immun-Blot^{TM} PVDF, USA) for antibody probing. Membranes were incubated for 60 min with blocking buffer (50 ml of phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin) and then with monoclonal antibody 8E2/2, raised in mouse to DnaK, at the recommended dilution of 1:1000 (Stressgen Bioreagents, Canada). Horseradish peroxidase conjugated donkey anti-mouse IgG was used as secondary antibody at the recommended dilution of 1:2500 (Affinity Bioreagents^{TM}, USA). Detection was with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate (DAB) in association with 0.01% (v/v) H_2O_2 in 0.1 M Tris-HCl (pH 7.6).

Results

Hsp producing bacteria promote survival of *Artemia* larvae upon *Vibrio* exposure

Heat shocking bacterial strain CAG 626 enhanced its ability to protect *Artemia* larvae from *Vibrio* infection by approximately two-fold (Fig. 6.1). However, CAG 629, which in

contrast to CAG 626 does not react to heat stress by increasing Hsp production, failed to respond similarly (Figs. 6.1, 6.2A, B). Strain P0, transformed with empty vector only, protected *Artemia* larvae against *Vibrio* challenge slightly better than did either CAG 626 or CAG 629, perhaps due to its higher nutritional value, but protection was not increased by arabinose induction (Table 6.2, Fig. 6.1).

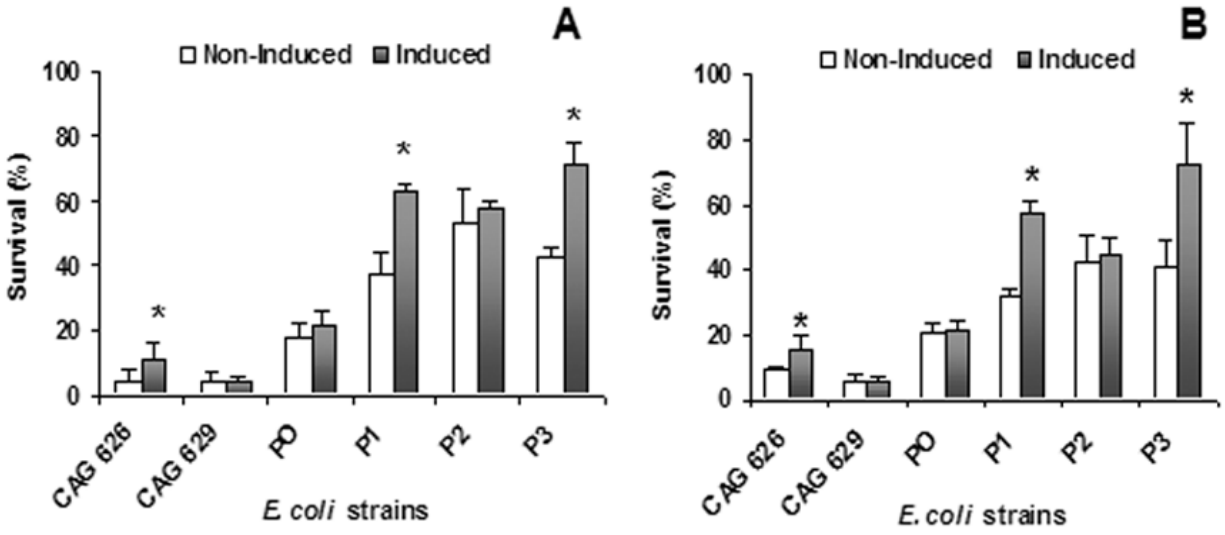

Fig. 6.1. Survival of *Artemia* larvae after challenge with *V. campbellii. Artemia* larvae fed once with bacteria at 1 x 10^7 cells/ml were incubated for 6 h prior to challenge with *V. campbellii* and survival was determined 36 h after challenge. Bacterial strains were either non-induced or they were induced by heat shock (CAG 626, CAG 629) and arabinose (P0, P1, P2 and P3). Replicate experiments are labelled A and B, and standard deviations (mean ± S.D.) are indicated. *, indicates significant differences between non-induced and induced larvae ($p<0.05$).

In contrast, strain P0 was less effective than strains P1, P2 and P3, all of which express Hsp cDNAs, in shielding *Artemia* against *Vibrio* challenge (Figs. 6.1, 6.2C, D). The effect of strain P2 on larval survival, although greater than P0 and similar to P1 was not enhanced by arabinose induction. Protection of *Artemia* larvae by strains P1 and P3, both of which contain plasmids that encode DnaK-DnaJ-grpE, was high, and in contrast to strain P2 which encodes GroES and GroEL, protection was improved by arabinose induction, with strain P3 giving the highest overall survival (Fig. 6.1).

Table 6.2 - Nutritional value of bacterial strains

Bacteria strains	A				B			
	Non-Induced		Induced		Non-Induced		Induced	
	Live larvae (%)	Length (mm)	Live larvae (%)	Length (mm)	Live larvae (%)	Length (mm)	Live larvae (%)	Length (mm)
CAG 626	44 ± 4^{cd}	0.8 ± 0.1^{b}	$59 \pm 6^{c*}$	0.8 ± 0.1^{b}	51 ± 6^{b}	0.8 ± 0.1^{b}	51 ± 4^{b}	0.8 ± 0.1^{a}
CAG 629	27 ± 3^{de}	0.8 ± 0.1^{b}	38 ± 2^{d}	0.8 ± 0.1^{b}	22 ± 3^{c}	0.8 ± 0.1^{b}	21 ± 3^{c}	0.8 ± 0.1^{a}
P0	62 ± 6^{bc}	1.0 ± 0.1^{a}	66 ± 5^{bc}	1.0 ± 0.1^{a}	61 ± 3^{b}	1.0 ± 0.1^{a}	63 ± 8^{b}	1.0 ± 0.1^{a}
P1	87 ± 10^{ab}	0.9 ± 0.1^{ab}	91 ± 8^{a}	0.9 ± 0.1^{a}	84 ± 7^{a}	1.0 ± 0.1^{ab}	84 ± 3^{a}	1.0 ± 0.1^{a}
P2	87 ± 13^{ab}	1.0 ± 0.1^{a}	85 ± 8^{ab}	1.0 ± 0.1^{ab}	87 ± 12^{a}	1.0 ± 0.1^{b}	90 ± 2^{a}	0.9 ± 0.1^{a}
P3	88 ± 11^{a}	1.0 ± 0.2^{ab}	93 ± 9^{a}	0.9 ± 0.1^{ab}	93 ± 2^{a}	1.0 ± 0.1^{b}	91 ± 1^{a}	1.0 ± 0.1^{a}

Artemia were fed once with bacteria at 1 x 10^7 cells/ml and following 2 days incubation the number and length of live larvae were determined. Replicate experiments are labeled A and B. Bacteria were either non-induced or they were induced by heat shocking (CAG 626 and CAG 629) or with arabinose (P0, P1, P2, P3); length, average length of larvae. Survival of non-fed *Artemia* was 9 ± 5% for A and 13 ± 5% for B. Standard deviation (mean ± S.D.) is included for each average. Values in the same column for each experiment showing the same superscript letter are not significantly different (p>0.05). *, indicates significant difference between non-induced and induced samples.

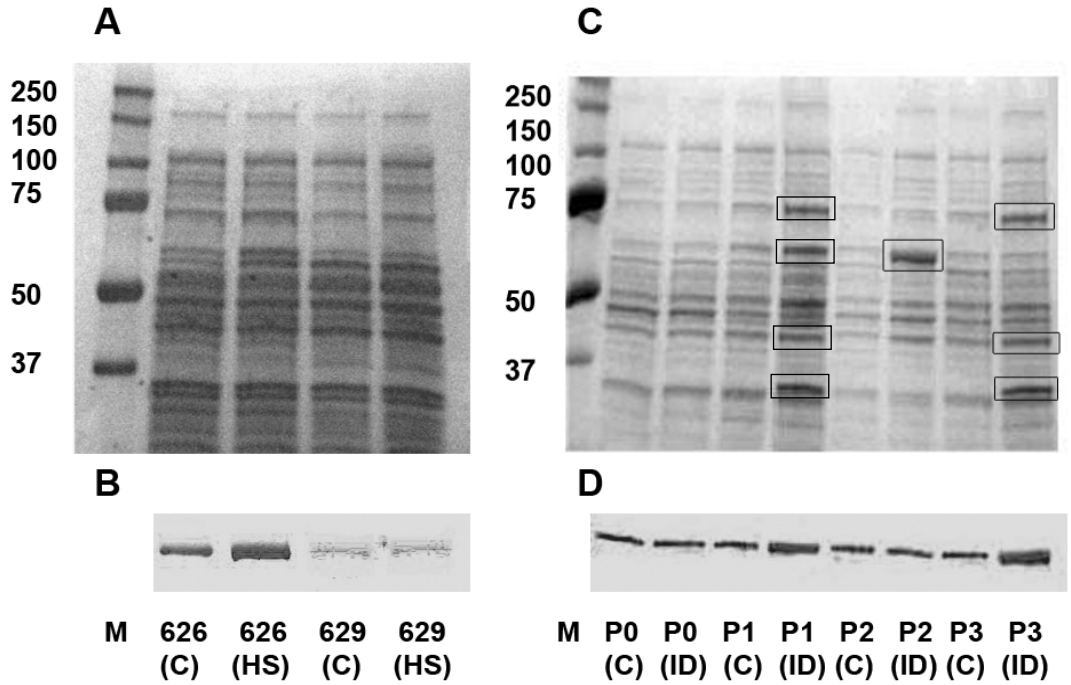

Fig. 6.2. Induction of Hsp synthesis. Bacterial proteins were resolved in SDS polyacrylamide gels and either stained with Coomassie Biosafe (A, C) or transferred to polyvinylidene fluoride membranes and probed with antibody to DnaK (B, D). C, non-induced bacteria; HS, heat shocked; ID, arabinose induced. Fifty μg of protein was loaded in each lane. Molecular mass standards (M) in kDa are on the left. Boxes, recombinant DnaK, DnaJ, grpE and GroEL, appearing from top to bottom.

Augmentation of protection against *Vibrio* challenge upon arabinose induction by strains P1 and P3, but not P2, suggested DnaK, DnaJ and/or grpE were responsible. Electrophoresis of bacterial extracts in SDS polyacrylamide gels and staining with Coomassie blue demonstrated prominent polypeptides of 20, 40, 60 and 70 kDa in extracts from arabinose-induced strain P1, as was true for strain P3 except the 60 kDa polypeptide representing GroEL was lacking (Fig. 6.2C). Polypeptides of 20, 40 and 70 kDa were representative of the Hsps encoded by the cDNAs used to transform strains P1 and P3. Immunoprobing of bacterial protein extracts transferred to polyvinylidene fluoride membranes demonstrated that the 70 kDa protein enhanced by arabinose induction in strains P1 and P3 is DnaK (Fig. 6.2D). Other Hsps were not identified by this method.

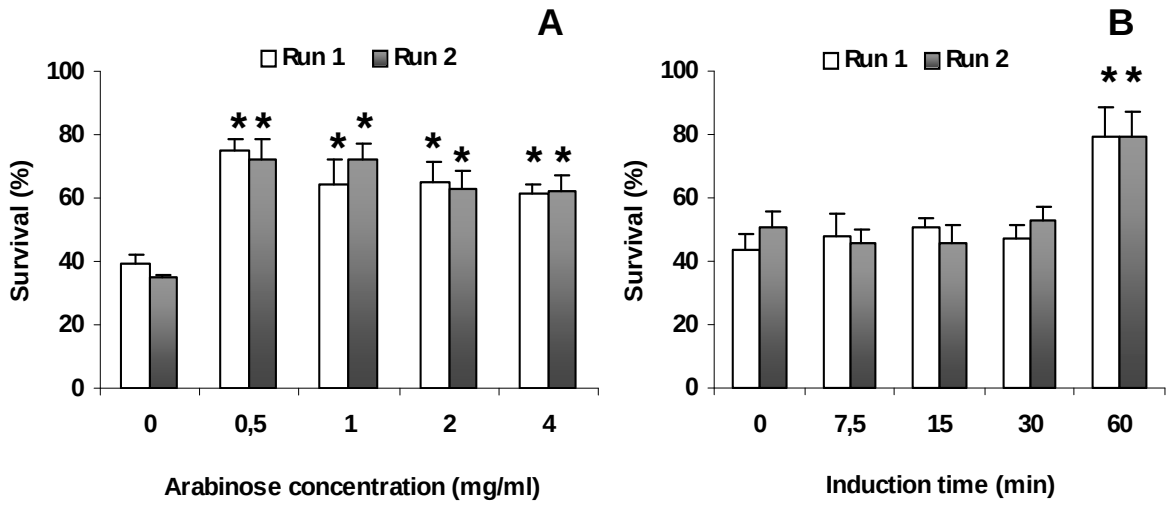

Fig. 6.3. Incubation with arabinose enhanced the ability of strain P3 to protect *Artemia*. (A) *E. coli* strain P3 was induced with different concentration of L-arabinose for 1 h and then tested for the ability to protect *Artemia* larvae against *V. campbellii*. (B) *E. coli* strain P3 was induced with 0.5 mg/ml L-arabinose for different times and tested for the ability to protect *Artemia* larvae against *V. campbellii*. Replicate experiments are labelled Run 1 and Run 2. Standard deviations (mean ± S.D.) are indicated. *, indicates significant differences between control and induced larvae ($p<0.05$).

Because greater protection may have been due simply to nutritional parameters the effects of feeding bacteria on the growth of *Artemia* larval were tested. Mortality was high after 48 h when *Artemia* were not fed, with only 9% and 13% of larvae alive in respective replicate experiments. Feeding *Artemia* with *E. coli* significantly increased the number of live

larvae with bacterial strains varying in nutritional quality (Table 6.2). For example, in comparison to strain CAG 626, feeding with either non-induced or induced strain P0 gave a slight but insignificant increase in live larvae, whereas differences upon feeding with CAG 629 and P0 were significant. Larval length was significantly greater in most cases when comparing P0 to either CAG 626 or CAG 629 ($p<0.05$). The number of live larvae increased substantially and length modestly when *E. coli* strains, P1, P2 and P3 were used as feed, although induction boosted neither value (Table 6.2). Thus, bacteria were used as food by *Artemia* larvae but differences in nutrient value accounted, at best, for only minor differences in *Vibrio* resistance.

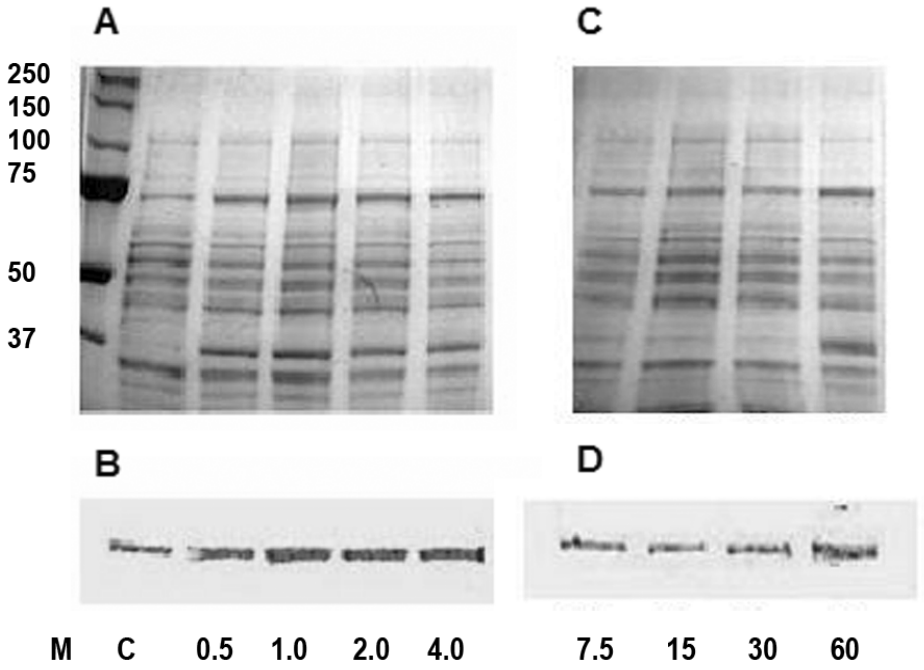

Fig. 6.4. Arabinose induced over-expression of DnaK by strain P3. Protein from *E. coli* strain P3 induced with L-arabinose was resolved in SDS polyacrylamide gels and either stained with Coomassie Biosafe (A, C) or transferred to polyvinylidene fluoride membranes and probed with antibody to DnaK (B, D). C, non-induced; 0.5 – 4.0, arabinose induction respectively at 0.5 – 4.0 mg/ml; 7.5 - 60, induction with 0.5 mg/ml arabinose respectively for 7.5 – 60 min. Fifty μg of bacterial protein was loaded in each lane. Molecular mass standards (M) in kDa are on the left.

DnaK synthesis by strain P3 correlates with protection against *V. campbellii*

Because previous research showed that Hsp70 expression coincided with *Vibrio* resistance in *Artemia* (Yeong et al., 2007), and the greatest protective activity was obtained in

initial experiments with strain P3, which exhibited enhanced DnaK synthesis, the correlation between survival and DnaK was investigated further. A significant increase (p<0.05) in survival occurred during challenge tests when *Artemia* larvae were fed *E. coli* strain P3 induced with L-arabinose in the range of 0.5 to 4.0 mg/ml, with maximal protection at the lowest sugar concentration (Fig. 6.3A). Using 0.5 mg/ml arabinose, an induction period of at least 60 min was required to boost survival of *Artemia* larvae upon *Vibrio* exposure (Fig. 6.3B). As revealed by SDS polyacrylamide gel electrophoresis and immunostaining of western blots, increased DnaK production correlated with greater larval survival (Fig. 6.4).

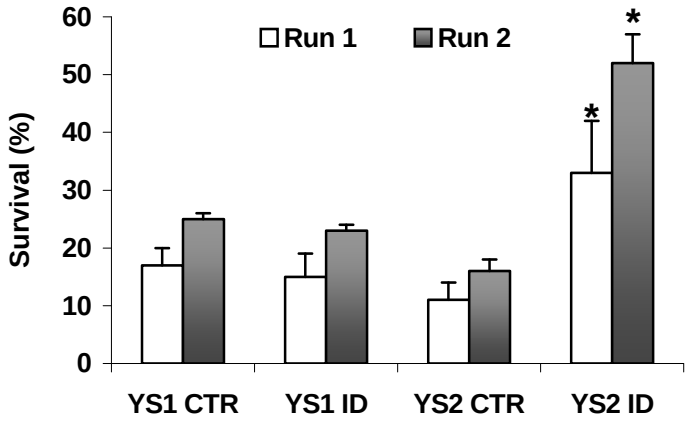

Fig. 6.5. Strain YS2 over-producing DnaK protects *Artemia* against *V. campbellii*. *Artemia* larvae were fed once with bacterial strains YS1 and YS2 at 1 x 10^7 cells/ml and incubated 6 h before challenge with *V. campbellii* for 24 h. CTR, non-induced; ID, arabinose induced. Replicate experiments are labelled A and B. Standard deviations (mean ± S.D.) are indicated. *, indicates significant differences between induced and non-induced bacteria.

DnaK enhances protection of *Artemia* larvae against *Vibrio* infection

Survival of *Artemia* larvae fed either induced or non-induced strain YS1 was low upon challenge with *V. campbellii*, results similar to those obtained with non-induced YS2 (Fig. 6.5). In contrast, a significant increase in survival (p<0.05) occurred when larvae fed with arabinose-induced YS2 were exposed to *V. campbellii* (Fig. 6.5). DnaK was up-regulated in arabinose-induced YS2, yielding a polypeptide of approximately 83 kDa visible in Coomassie-stained SDS polyacrylamide gels (Fig. 6.6A) and on western blots probed with

antibody to DnaK (Fig. 6.6B). The increase in molecular mass of approximately 13 kDa, as compared to the normal mass of DnaK, was due to the amino-terminal incorporation of thioredoxin encoded by the TOPO® cloning vector. The high molecular mass DnaK was not detected in extracts prepared from strain YS1 and non-induced strain YS2. The nutritional value of induced strain YS2 was significantly different from non-induced in experiment A but not B (Table 6.3), but in both cases the DnaK containing bacteria provided approximately 3-fold increase in survival as compared to non-induced bacteria (Fig. 6.5). These data indicate that even though the nutritional value of induced and non-induced bacteria may vary these differences do not account for enhanced survival. Clearly, protection against *Vibrio* challenge is improved by DnaK, although the possibility that other Hsps have this capability is not discounted.

Table 6.3 - Nutritional value of bacterial strains YS1 and YS2

Bacteria strains	A				B			
	Non-induced		Induced		Non-induced		Induced	
	Live larvae (%)	Length (mm)	Live larvae (%)	Length (mm)	Live larvae (%)	Length (mm)	Live larvae (%)	Length (mm)
YS1	33 ± 8	1.2 ± 0.3[a]	39 ± 3	1.1 ± 0.2[a]	29 ± 5	1.0 ± 0.1[a]	32 ± 4	1.1 ± 0.1[a]
YS2	39 ± 4	1.2 ± 0.3[a]	51 ± 2*	0.9 ± 0.2[a]	29 ± 5	0.9 ± 0.1[a]	34 ± 5	0.9 ± 0.1[a]

Artemia were fed with bacteria strains YS1 and YS2 at 1 x 10^7 cells/ml and following 2 days incubation the number and length of live larvae were determined. Replicate experiments are labeled A and B. Bacteria were induced with arabinose. Survival of non-fed *Artemia* was 15 ± 1% for A and 16 ± 3% for B. Standard deviation (mean ± S.D.) is included for each average. *, indicates significant differences between non-fed and fed larvae (p<0.05).

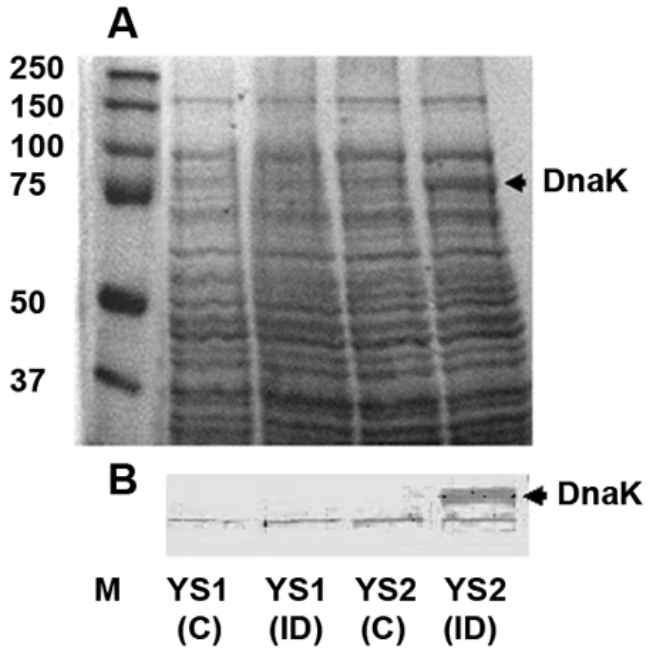

Fig. 6.6. Synthesis of DnaK in strain YS2. Protein extracts from *E. coli* strains YS1 and YS2 were resolved in SDS polyacrylamide gels and either stained with Coomassie Biosafe (A) or transferred to polyvinylidene fluoride membranes and probed with antibody against DnaK (B). C, non-induced; ID, induced. Fifty μg of protein was loaded in each lane. Molecular mass standards (M) in kDa are on the left. Labelled arrow, recombinant DnaK.

Discussion

Artemia larvae exposed solely to *V. campbellii* experienced significant mortality, in agreement with other results where this bacterium was used as pathogen (Marques et al., 2006a; Defoirdt et al., 2006, 2007; Soltanian et al., 2007; Yeong et al., 2007). The *V. campbellii* infection route is unknown, but the closely related pathogenic species *V. proteolyticus* disrupts microvilli and epithelial junctions, destroying tissues within the *Artemia* body cavity (Verschuere et al., 2000b)**.** To further examine *Vibrio* infection and test the hypotheses that Hsp70 safeguards *Artemia* against *V. campbellii* (Yeong et al., 2007), challenge tests were performed subsequent to feeding gnotobiotic larvae with bacteria over-expressing prokaryotic Hsps. The best survival was obtained initially with the strains P1, P2 and P3. P1 and P3 effectiveness was enhanced upon Hsp induction, suggesting resistance is associated with DnaK, DnaJ and grpE. Strain P2, transformed with cDNA encoding GroES-

GroEL also improved larval survival as compared to P0 but this activity was indifferent to induction, reflecting a change not involving inducible Hsps. Varying induction conditions for strain P3, which conferred the highest survival in initial challenge tests, showed that enhanced protection of *Artemia* larvae and Hsp synthesis are correlated. A definitive role for DnaK in promoting survival was demonstrated by feeding strain YS2 to *Artemia* larvae prior to *Vibrio* exposure.

Substantial evidence indicates that Hsps modulate the innate immune system, consequently contributing to disease resistance. For example, application of Hsps *in vitro* induced phagocytes and granulocytes to release reactive oxygen species, cationic peptides, lysozyme and cytokines (Jacquier-Sarlin et al., 1994; Basu et al., 2002). Additionally, macrophages and neutrophils were stimulated to produce nitric oxide synthase (Panjwani et al., 2002), nitric oxide (Campisi and Fleshner, 2003), tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 (Asea et al., 2000; Campisi and Fleshner, 2003; Johnson and Fleshner, 2006), all extracellular suppressors of infection. Hsps also activate Toll like receptors which transduce inflammatory signals to innate immune cells and promote resistance against disease and infections (Asea et al., 2002; Vabulas et al., 2002). Furthermore, exogenous administration of Hsps up-regulates two major macrophage/monocyte differentiation markers (Edgington, 1995).

In mammals, where the molecular mechanisms of the innate immune system are well defined, bacterial pathogens which produce Hsps when stimulated by fever activate host immunity (Hasday and Singh, 2000). In contrast, the way in which exogenous administration of prokaryotic Hsps, and particularly the 70 kDa DnaK, enhances protection in *Artemia* remains unclear. However the results in this paper indicate that bacterial Hsps stimulate the *Artemia* innate immune response, playing a significant role in defending larvae against pathogenic stress. Interestingly, endogenous Hsp70 accumulation induced by abiotic stress in

gnotobiotic larvae reduced *Vibrio* colonization of *Artemia* (Yeong et al., in press) and enhanced resistance to infection (Yeong et al., 2007). In comparison, increasing Hsp70 by short-term hyperthermic stress correlated with survival enhancement and attenuation of gill-associated virus (GAV) replication in the black tiger prawn, *Penaeus monodon* (de la Vega et al., 2000). Moreover, stress induced synthesis of small heat shock proteins and Hsp90 triggers *C. elegans* immunity to pathogenic *Pseudomonas aeruginosa*. The mechanism may involve a conserved pathway including heat shock transcription factor-1 (HSF-1) and the associated DAF2/DAF-16 pathway which regulates aging and immunity in nematodes (Singh and Aballay, 2006).

Infection by luminescent vibrios causes serious damage during the rearing of molluscs, finfish, lobsters and shrimp (Pass et al., 1987; Alvarez et al., 1998; Lavilla-Pitogo et al., 1998; Diggles et al., 2000; Austin and Zhang, 2000; Defoirdt 2007) and as a consequence aquaculture systems suffer devastating losses (Bachère, 2003). Antibiotics are used to overcome disease, but this is expensive, produces resistant pathogens and contaminates the environment (Hameed and Balasubramaniam, 2000), placing other animals, including humans, in jeopardy. There is an urgent need to develop prophylactic measures with reduced environmental impact and the use of Hsp over-producing bacteria to enhance disease resistance, as shown in this study, is very promising. Moreover, that bacterially produced Hsps protect *Artemia* larvae from *Vibrio* infection suggests, by comparison to other organisms that an immunostimulatory mechanism is operating, an intriguing possibility.

Acknowledgements

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CHAPTER 7

Ingestion of bacteria over-producing DnaK attenuates *Vibrio* infection of gnotobiotic *Artemia franciscana* larvae

Yeong, Y. S., Dhaene, T., Defoirdt, T., Boon, N., MacRae, T. H., Sorgeloos, P. & Bossier, P.

Abstract

Feeding of bacterially encapsulated heat shock proteins (Hsps) to invertebrates is a novel way to limit *Vibrio* infection. As an example, ingestion of *Escherichia coli* over-producing prokaryotic Hsps significantly improves survival of gnotobiotically cultured *Artemia* larvae upon challenge with pathogenic *Vibrio campbellii*. The relationship between Hsp accumulation and enhanced resistance to infection may involve DnaK, the prokaryotic equivalent to Hsp70, a major molecular chaperone in eukaryotic cells. In support of this proposal, heat stressed bacterial strains LVS 2 (*Bacillus sp*), LVS 3 (*Aeromonas hydrophila*), LVS 8 (*Vibrio sp*), GR 8 (*Cytophaga sp*) and GR 10 (*Roseobacter sp*) were shown in this work to be more effective than non-heated bacteria in protecting gnotobiotic *Artemia* larvae against *V. campbellii* challenge. Immunoprobing of western blots and quantification by ELISA revealed that the amount of DnaK in bacteria and their ability to enhance larval resistance to infection by *V. campbellii* are correlated. Although the function of DnaK is uncertain it may improve tolerance to *V. campbellii* via immune stimulation. This possibility is currently under investigation and it promises to yield findings of fundamental importance with applications in aquaculture, a major method of food production.

Introduction

The brine shrimp *Artemia*, found worldwide in extreme environments, is used as a model organism for study of the stress response (Clegg et al., 2000a, b; Frankenberg et al., 2000, MacRae, 2003), aquatic diseases (Verschuere et al., 2000; Soto-Rodriguez, 2003; Marques et al., 2005; Yeong et al., 2007) feed quality (Marques et al., 2004a, b) and probiont characteristics (Marques et al., 2005, 2006a). As one example, a recently established gnotobiotic system, where larvae are hatched, grown in axenic conditions and exposed to known bacteria, has facilitated research on host-microbe interactions, particularly as they relate to *Artemia* larvae (Marques et al., 2006b). Among the topics investigated were the contribution of bacteria toward immune stimulation in *Artemia*, the role of bacteria as a food source for this crustacean, and the influence of bacteria on the nutritional properties of *Artemia* used as feed in aquaculture (Yasuda and Taga, 1980; Hessen and Anderson, 1990; Gorospe et al., 1996; Marques et al., 2005).

Physiological stress promotes synthesis of intracellular heat shock proteins (Hsps), often referred to as stress proteins or molecular chaperones, and they influence the synthesis, structure, localization and function of other cell proteins (Parsell and Lindquist, 1993). Members of the Hsp70 family, a prominent group of molecular chaperones, are robustly induced when cells experience temperature variation, oxygen deprivation, nutritional deficiency and infection (Morimoto, 1998; Feder and Hofmann, 1999). Hsp70 occurs in all types of eukaryotic organisms and it has approximately 60% similarity to DnaK, the prokaryotic Hsp70 equivalent (Pockley, 2003). As an unexpected finding, extracellular Hsp70, in combination with other Hsps, is thought to modulate peptide positioning on cell surfaces, thus enhancing immune recognition of aberrant cells (Athman and Philpott, 2004; Johnson and Fleshner, 2006). Of interest in this regard is research on economically important organisms used in aquaculture such as *Penaeus monodon* (de la Vega et al. 2006) and

Artemia franciscana (Yeong et al., 2007; in press), where Hsp70 is implicated in protection against pathogenic microbes, perhaps by stimulation of the immune response.

Aquaculture, an important source of human nutrition, is the fastest growing food production sector in the world, increasing about 8.8 % annually during the past decade and significantly supplementing capture fisheries (FAO, 2006). With this growth is the increased use of live food such as *Artemia* larvae. High nutritional properties, including those contributed by lipids and unsaturated fatty acids, make newly hatched *Artemia* larvae particularly suitable as a major starter diet in the rearing of farmed finfish and crustacean larvae. However, this advance necessitates rearing *Artemia* larvae under conditions where infection by bacteria and other pathogens must be controlled. In relation to this requirement, endogenous Hsp70 (Yeong et al., 2007), and exogenously supplied DnaK synthesized in transformed *Escherichia coli*, were shown to protect *Artemia* larvae from *Vibrio* infection (refer to chapter 6), perhaps by immune stimulation. The objective of the present study was, therefore, to better understand how DnaK protects *Artemia* larvae from bacterial infection.

Materials and Methods

Induction of bacterial Hsp synthesis

Bacillus sp. (LVS 2), *Aeromonas hydrophila* (LVS 3), *Vibrio* sp. (LVS 8), *Cytophaga* sp. (GR 8), *Roseobacter* sp. (GR 10) and *Vibrio campbellii* strain LMG 21363 (Table 7.1) were stored in 40% glycerol at –80°C. Bacteria were grown at 28°C for 48 h on marine agar and then to log phase in marine broth 2216 (Difco Laboratories, Detroit, Mich.) by overnight incubation at 28°C. Heat shock protein synthesis was induced in bacteria, with the exception of *V. campbellii*, by a 30 min exposure to 38°C at a Δt of 4°C/min in a preheated water bath. The bacterial strains were then transferred individually to sterile tubes, centrifuged at 2200 *g* for 15 min at 28°C and suspended in filtered autoclaved sea water, used throughout the study.

The turbidity of bacterial cultures was determined spectrophotometrically at 550 nm and these values were used to determine bacterial numbers according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an optical density of 1.000 corresponds to 1.2 x 10^9 cells/ml.

Table 7.1. Bacterial strains

Bacteria	Strain	References
Bacillus sp.	LVS 2	Verschuere et al., 1999; 2000b Marques et al., 2005
Aeromonas hydrophila	LVS 3	Verschuere et al., 1999; 2000b Marques et al., 2005
Vibrio sp.	LVS 8	Verschuere et al., 1999; 2000b Marques et al., 2005
Cythophaga sp.	GR 8	Marques et al., 2005
Roseobacter sp.	GR 10	Marques et al., 2005
Vibrio campbellii	LMG 21363	Soto-Rodriguez et al., 2003a Gomez-Gil et al., 2004

Vibrio challenge of _Artemia_ larvae

Axenic *Artemia* larvae were obtained by cyst decapsulation (Sorgeloos et al., 1986) followed by incubation at 28°C for 24 h in sea water (Yeong et al., 2007). Larvae developing within the next 4-6 h, and thus with the ability to ingest bacteria, were harvested and incubated for 6 h with either heat shocked or non-stressed bacteria at approximately 1 x 10^7 cells/ml. Challenge tests were then performed by adding pathogenic *V. campbellii* at 1 x 10^7 cells/ml and incubating with constant agitation and light for 36 h prior to recovery of swimming larvae, fixation in Lugol's solution, and counting. Survival percentage was calculated as N_t x 100/ N_o where N_t and N_o are final and initial numbers of live larvae, respectively. The increase in survival conferred by each bacterial strain upon heating was calculated as ($S_f - S_i$) x 100/ S_i where S_f and S_i are percentage survival of *Artemia* fed heated

and non-heated bacteria, respectively. Axenity was confirmed by plating 100 µl of culture medium containing *Artemia*, but receiving no bacteria, on marine agar and incubating 5 days at 28°C (Marques et al., 2004a). Results were discarded if contamination was found.

Nutritional value of bacteria

The nutritional value of bacterial strains (Table 7.1) was determined by a single feeding of 1 x 10^7 bacteria/ml to axenically cultured *Artemia*. Viability, as survival percentage, was determined after 2 days as just described. Fixed nauplii were measured by using a dissecting microscope equipped with a drawing mirror, a digital plan measure, and Artemia 1.0® software (courtesy of Marnix Van Domme).

Statistics

Larval survival (%) in challenge and nutritional tests were ArcSin to satisfy normality and homocedasticity requirements while length measurements were either logarithmic or square root transformed as necessary. Differences in larval survival and lengths were investigated by performing analysis of variances (ANOVA) and Tukey's multiple comparisons range using statistical analysis software SPSS® version 11.5 for Windows®.

Immunodetection of Hsps

Protein extraction was as described (Yeong et al., 2007) except bacteria were homogenized in the presence of 0.1 mm diameter glass beads in ice cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) (Clegg et al., 2000a) containing protease inhibitors (Catalogue # P8465, Sigma-Aldrich, Inc. USA) at the highest recommended level. Protein concentrations were determined by the Bradford method and equal amounts of protein were loaded in each gel lane.

Bacterial proteins resolved in 10% SDS polyacrylamide gels (Laemmli, 1970) were either stained with Coomassie Biosafe (BioRad™ Laboratories, USA) or transferred to polyvinylidene fluoride membranes (BioRad Immun-Blot™ PVDF, USA) before incubating 60 min with blocking buffer (phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin). Membranes were incubated for 1 h at room temperature with rabbit polyclonal antibody raised against the ATPase domain of *E. coli* DnaK (Bucca et al., 2000), a generous gift from Dr. Bernd Bukau, ZMBH, Germany. Subsequent to washing the membrane was incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (Gentaur BVBA, Belgium) for 1 h, washed and exposed to 0.7 mM diaminobenzidine tetrahydrochloride dihydrate and 0.01% (v/v) H_2O_2 in 0.1 M Tris-HCl (pH 7.6) to detect antibody-reactive proteins. The 70 kDa protein detected with this antibody in various bacterial strains is subsequently referred to as DnaK.

DnaK quantification by ELISA

DnaK was quantified with the Sensiflex™ ELISA Development Kit (Molecular Probes, Invitrogen USA) following manufacturer's instructions. Approximately 50 µg of bacterial protein in 100 µl of sodium bicarbonate buffer, pH 9.3, was added to each well of a 96-well round-bottom polystyrene plate (Nunc-Immunoplate Maxisorp, Denmark) and incubated at 4°C overnight. Plates were washed three times with phosphate-buffered saline containing 0.1% Tween 20 (PBST) and blocked 4 h at 28°C with PBS containing 5% BSA. One hundred µl of rabbit polyclonal antibody raised against *E. coli* DnaK was added to each well, and plates were incubated for 30 min at 37°C prior to washing. Goat anti-rabbit IgG (H+L) with β-lactamase TEM-1 conjugate was then added and after 30 min at 28°C the plates were washed. Detection of antibody reactivity was by incubation for 30 min with 100 µl of 0.9 mM Fluorocilin™ Green reagent. Fluorescence intensity was determined at 495 nm

excitation and 525 nm emission in a Tecan® Infinite M200 ELISA microplate reader. A standard curve, constructed by use of recombinant DnaK (Prospec-Tany TechnoGene Ltd, Israel), was used to convert sample absorbance to DnaK content with values expressed as *E. coli* equivalent. All experiments were done in duplicate.

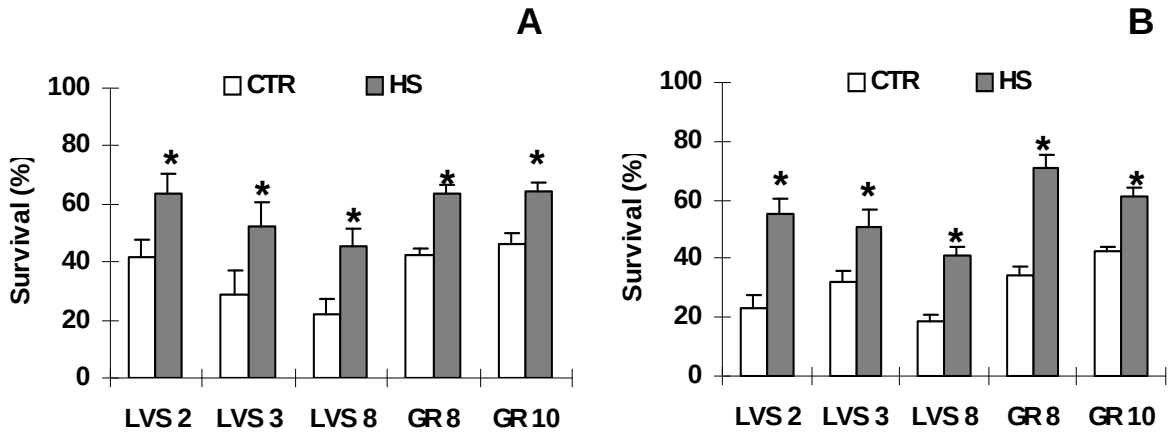

Fig. 7.1. Survival of *Artemia* larvae after challenge with *V. campbellii*. Larvae were fed different bacterial strains at 1 x 10^7 CFU/ml prior to challenge tests. Experimental replicates are labeled A and B and the averages of results are given with standard deviations (mean ± S.D.). CTR, bacteria not heated; HS, bacteria were heated. Values in the same column for each experiment showing the same superscript letter are not significantly different (p>0.05). *, indicates significant differences in larval survival between control and heat shock treatments.

Results

Heating of bacteria enhances their ability to protect *Artemia* larvae against *V. campbellii*

Feeding *Artemia* larvae heat shocked as opposed to non-heated bacteria significantly (p<0.05) increased survival upon exposure to *V. campbellii* (Fig 7.1). Strain LVS 8, although conferring the lowest protection on *Artemia* larvae, whether or not bacteria were heated, nonetheless, greatly improved protection when heat shocked bacteria were employed. In contrast, GR 10, which gave the best protection of any strain prior to heat shock, provided the least enhancement of larval protection upon heating. Increases in protection granted by heating strain LVS 3 were between that bestowed by heat shocking LVS 8 and GR 10,

whereas the effects of LVS 2 and GR 8 were variable, although both provided significantly increased protection against *Vibrio* infection upon heating (Table 7.2).

Table 7.2. Feeding with heat stressed bacteria increases *Artemia* resistance to *V. campbellii*

Bacteria strains	A Increase in survival (%)	B Increase in survival (%)
LVS 2	50 ± 7	139 ± 10
LVS 3	79 ± 13	59 ± 6
LVS 8	105 ± 11	116 ± 5
GR 8	47 ± 2	109 ± 5
GR 10	41± 4	42 ± 1

Artemia larvae were incubated in replicate experiments A and B with non-heated and heat stressed bacteria at 1 x 10^7 cells/ml for 6 h prior to challenge with *V. campbellii* and then the number of surviving larvae were determined. The percentage increase in survival was calculated as described in the Materials and Methods and the results are presented as mean ± S.D.

Heat shock increased DnaK production by bacteria

Coomassie stained gels containing protein extracts from heated and non-heated bacteria of single strains were similar to one another although LVS 2 exhibited a slight increase in a 70 kDa polypeptide upon heating (Fig 7.2A). Immunoprobing of western blots with an antibody to DnaK revealed a single reactive polypeptide of approximately 70 kDa in all strains except LVS2 where two 70 kDa polypeptides were detected. Staining intensity of the antibody reactive 70 kDa polypeptides was greater in protein extracts from heat shocked versus non-heated bacteria (Fig 7.2B).

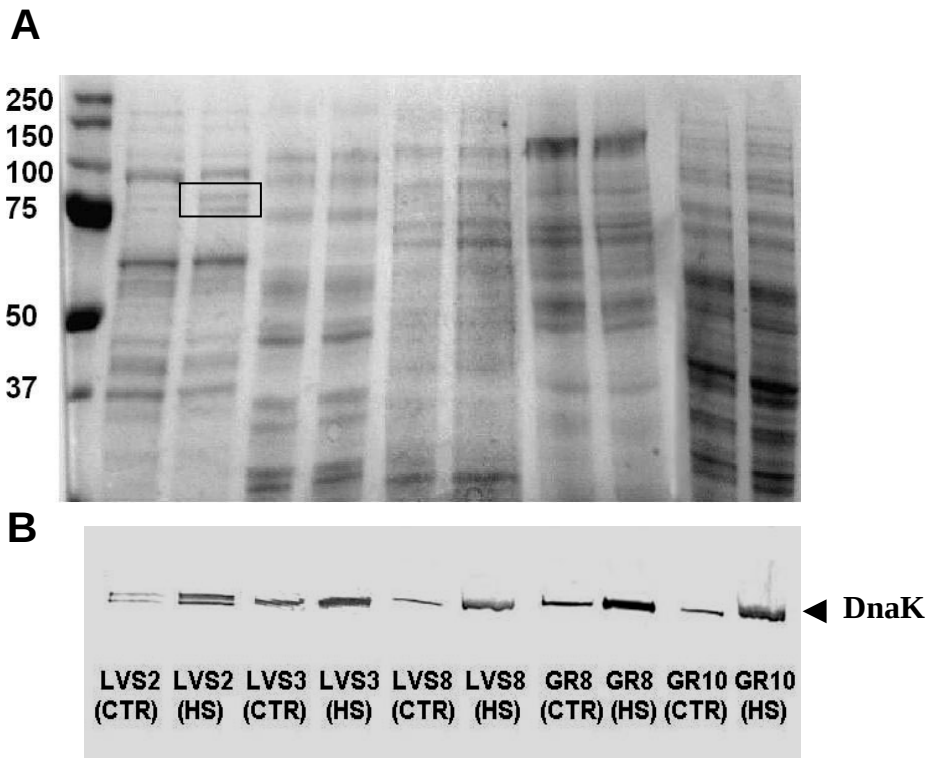

Fig. 7.2. Heat shock stimulates DnaK synthesis. Bacterial protein extracts were electrophoresed in SDS polyacrylamide gels and either stained with Coomassie Biosafe (A) or blotted to polyvinylidene fluoride membranes and incubated with antibody to DnaK (B). Approximately 50 μg of protein was loaded in each lane; CTR, non-heated bacteria, HS, heated bacteria. Molecular mass standards in kDa are on the left. Box, 70 kDa polypeptide.

Additionally, quantification by ELISA demonstrated that DnaK increased from 2.0 - 2.3 fold in heated bacteria (Fig. 7.3). The higher amounts of DnaK in heated as opposed to non-stressed bacteria correlated with the enhanced ability of these bacteria to promote survival of *Artemia* larvae.

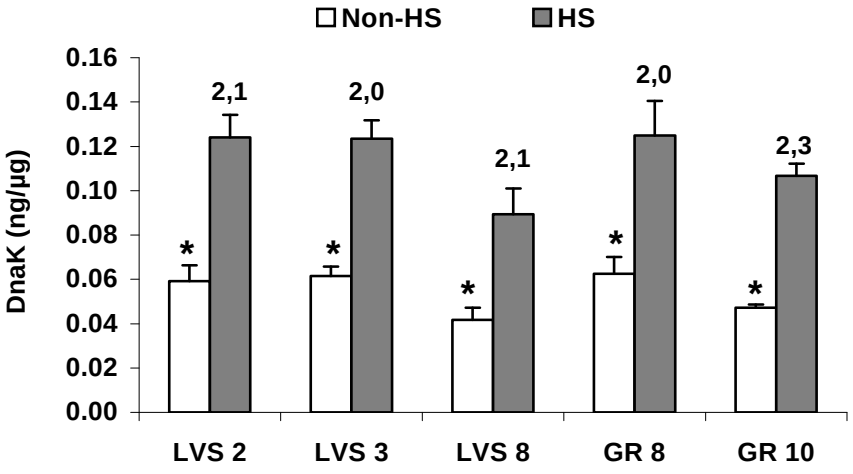

Fig. 7.3. Heating increases bacterial DnaK content. The amount of DnaK was determined in non-heated (Non-HS) and heated (HS) bacteria by ELISA and presented as mean ± SD (ng/µg). The fold-increase in DnaK upon heating each bacterial strain is shown above the bars. *, indicates significant difference in DnaK levels between non-heated and heated bacteria.

Larval resistance to *Vibrio* infection is not determined by the nutritional value of bacteria

Mortality was high in replicate experiments, even in the absence of *Vibrio* exposure, when *Artemia* larvae were starved, with only 9 ± 5% and 13 ± 5% of larvae alive in replicate experiments after 48 h. Conversely, incubating larvae with any of the bacterial strains employed in this study, except *V. campbellii*, significantly increased the number of live larvae ($p<0.05$) (Table 7.3). Bacterial strains varied in nutritional quality. In comparison to LVS 8, feeding with both LVS 2 and LVS 3 substantially increased viable larvae, while moderate but insignificant increases were obtained with GR 8 and GR 10 ($p<0.05$). Differences in the nutritional values of heated versus non-heated bacteria were insignificant ($p>0.05$), except for LVS 8 and GR 8 in experiment A which gave slight but significant increases in viable larvae. In no case was the difference in nutritional value great enough to account for the enhanced survival conferred on larvae by feeding with heated versus non-heated bacteria. Regardless of heat exposure, the average length of *Artemia* larvae was highest when larvae were fed strain LVS 2 and lowest when LVS 8 was employed.

Generally, length variation was insignificant when comparing results obtained by feeding larvae with LVS 2 and other bacterial strains (p>0.05), although some differences were apparent (Table 7.3). Incubation with only *V. campbellii* killed all larvae.

Table 7.3. Nutritional value of bacteria

Bacteria strains	A				B			
	CTR		Heat shock		CTR		Heat shock	
	Live larvae (%)	Individual length (mm)	Live larvae (%)	Individual length (mm)	Live larvae (%)	Individual length (mm)	Live larvae (%)	Individual length (mm)
LVS 2	89 ± 6^{a}	1.3 ± 0.1^{a}	82 ± 7^{a}	1.3 ± 0.1^{a}	89 ± 1^{a}	1.2 ± 0.1^{a}	$79 \pm 1^{a*}$	1.3 ± 0.1^{a}
LVS 3	75 ± 8^{a}	1.2 ± 0.3^{ab}	81 ± 4^{a}	1.1 ± 0.2^{a}	72 ± 8^{b}	1.1 ± 0.1^{ab}	79 ± 3^{a}	1.1 ± 0.2^{ab}
LVS 8	35 ± 4^{b}	1.0 ± 0.1^{b}	$47 \pm 2^{b*}$	1.1 ± 0.1^{a}	49 ± 8^{c}	1.0 ± 0.1^{b}	43 ± 1^{c}	1.1 ± 0.2^{ab}
GR 8	52 ± 2^{b}	1.1 ± 0.2^{ab}	$43 \pm 4^{b*}$	1.2 ± 0.1^{a}	59 ± 8^{bc}	1.1 ± 0.1^{b}	59 ± 4^{b}	1.2 ± 0.2^{ab}
GR 10	48 ± 5^{b}	1.1 ± 0.2^{ab}	57 ± 7^{b}	1.1 ± 0.2^{a}	51 ± 6^{c}	1.1 ± 0.1^{b}	55 ± 3^{b}	1.1 ± 0.1^{b}

Artemia were incubated with bacteria at 1 x 10^7 cells/ml and live larvae were counted after 48 h. Experimental replicates are labeled A and B and results are shown as the average of three determinations with standard deviations (mean ± S.D.). Only 9 ± 5% and 13 ± 5% respectively of larvae were alive in replicates A and B when *Artemia* were not fed. Live larvae (%), survival after 48 h of larvae fed once with 1 x 10^7 CFU/ml bacteria; Individual length (mm), average length of larvae (n = 10) 48 h after one feeding with 1 x 10^7 CFU/ml bacteria; CTR, bacteria were not heat shocked; Heat shock, bacteria were heat shocked. Values in each column showing the same superscript letter are not significantly different (p>0.05). *, indicates significant difference in live larvae between treatments using heated and non-heated bacteria.

Discussion

Feeding with bacteria, apart from virulent strains such as *V. campbellii* (Soltanian et al., 2007; Yeong et al., 2007), *V. proteolyticus* (Marques et al., 2006a; Yeong et al., 2007) and *V. harveyi* (Soto-Rodriguez et al., 2003; Defoirdt et al., 2006a), enhances *Artemia* resistance against disease and infection. As one example, colonization of cultures with nine different live bacterial strains protected *Artemia* juveniles against *V. proteolyticus* CW8T2 (Verschuere et al., 1999). Moreover, dead bacteria contribute similar beneficial effects

suggesting as one possibility that enhanced tolerance of *Artemia* to virulent bacteria occurs through stimulation of the non-specific immune response (Verschuere et al., 2000b; Marques et al., 2006a).

In this study, feeding gnotobiotic *Artemia* with heat shocked bacteria significantly increased larval resistance against the detrimental effects of *V. campbellii*. Additionally, immunoprobing of western blots and protein quantification by ELISA demonstrated that enhanced protection is associated with increased DnaK accumulation in bacteria used as feed. Previously, feeding with *E. coli* over-expressing DnaK, produced under the control of its own promoter or a heterologous arabinose inducible promoter, was shown to boost *Artemia* survival approximately three-fold (refer to chapter 6). The present study extends these observations, demonstrating clearly that DnaK-producing bacteria other than *E. coli* confer protection upon *Artemia* larvae. Moreover, the protective effects obtained by feeding many different strains of heat shocked bacteria with increased DnaK suggest direct involvement of this protein.

The effects of beneficial bacteria in an aquaculture system can be explained by various mechanisms such as improvement of water quality, antagonism towards pathogens including competition for adhesion sites, enzymatic contribution to digestion in the host, and stimulation of the host immune response (Verschuere et al., 2000b; Farzanfar, 2006; Tinh et al., in press). How Hsp producing bacteria protect brine shrimp has yet to be determined, but there is compelling evidence that these proteins induce strong immunological responses in other organisms (Pockley, 2003). Bacterial Hsps robustly stimulate the production of pro-inflammatory cytokine in human monocytes (Galdiero et al., 1997) and induce interleukin-1 secretion from macrophages (Retzlaff et al., 1994). Additionally, extracellular Hsp70 increases production of inducible nitric oxide synthase and nitric oxide (Panjwani et al., 2002; Campisi and Fleshner, 2003), as well as tumor necrosis factor-α, interleukin (IL)-1β,

and IL-6 (Asea et al., 2000; Campisi and Fleshner, 2003) in macrophages and neutrophils (Johnson and Fleshner, 2006). Furthermore, phagocytes and granulocytes release lysozyme, reactive oxygen species and cationic peptides upon exposure to Hsps (Jacquier-Sarlin et al., 1994; Basu et al., 2002), all of which suppress infection.

There is accumulating evidence that stimulation of the innate immune response by Hsps protects invertebrates from disease and infection. For example, build up of Hsp70 after short-term hyperthermic stress correlates with attenuation of gill-associated virus (GAV) replication in the black tiger prawn, *Penaeus monodon* (de la Vega et al., 2000). Acting through a conserved pathway involving heat shock transcription factor-1 (HSF-1) and the associated DAF2/DAF-16 pathway, stress regulated small Hsps and Hsp90 trigger immunity in the nematode *C. elegans* against *Pseudomonas aeruginosa* (Singh and Aballay, 2006). Additionally, endogenous Hsp70 accretion enhances resistance of gnotobiotic *Artemia* larvae to *V. campbellii* and *V. proteolyticus* (Yeong et al., 2007; in press). In both studies, Hsps were proposed to activate the *Artemia* innate immune system, thus promoting recognition and destruction of pathogens by defensive mechanisms.

In this study, administration of heat-shocked bacteria enriched in DnaK represented an efficient strategy for bio-control of vibriosis. This observation has applied significance in aquaculture where disease associated with luminescent vibrios causes mass mortalities of cultured organisms and economic losses worldwide (Diggles et al., 2000; Austin and Zhang, 2006). Antibiotic therapy is used but this results in microbial resistance, tissue accumulation of antibiotic residues (Vadstein, 1997) and immuno-suppression (Hameed and Balasubramanian 2000; Smith et al., 2003), consequently posing serious threats to human health. Application of the results described in this paper has the potential to alleviate these types of problems. The current findings also comment upon the relationship between Hsps,

the innate immune response and bacterial resistance in invertebrates, all of fundamental and applied significance.

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CHAPTER 8

Discussion and Conclusions

The gnotobiotic *Artemia* test (GART) system entails exposure of larvae, hatched and grown axenically, to bacteria under known conditions. The GART system is used to study host-microbe interactions (Marques et al., 2004a, 2006a, b, c), nutritional and immunostimulatory properties of yeast (Marques et al., 2006a; Soltanian et al., 2007) and algae (Marques et al., 2004a, b), probiont characteristics (Marques et al., 2005), and the application of quorum sensing to prevent infections in commercially grown organisms (Defoirdt, 2007; Tinh, 2007). Here, the GART system was extended to test Hsps as disease control agents and to examine the *Artemia* stress response.

A non-lethal heat shock protocol was optimized in this study to protect *Artemia* against pathogenic *Vibrio* challenge. Specifically, larvae given a preconditioning treatment at 37°C for 30 min with 6 h recovery accumulated endogenous Hsp70 and exhibited enhanced survival when exposed to *V. campbellii* and *V. proteolyticus* challenge. Likewise, a combined hypo- and hyperthermic stress followed by recovery at ambient temperature induced Hsp70 synthesis and shielded *Artemia* larvae against *V. campbellii*. In both studies, survival upon *Vibrio* challenge was augmented two-fold. Enhanced survival and induction of Hsp70 production coincide, suggesting this and/or other Hsps are directly involved in protecting larvae from infection. Moreover, hypothermic stress only and osmotic stresses which did not induce Hsp70 failed to increase *Artemia* tolerance to *Vibrio* challenge, substantiating the crucial role of this protein in conferring resistance.

The mechanisms by which Hsps, and particularly Hsp70, protect against pathogenic vibrios are uncertain, although immune stimulation is possible, a proposal strengthened by the observation that *Artemia* with increased Hsp70 possessed lower bacterial loads in challenge tests. Hsps regulate the invertebrate innate immune response and as one example, the heat-induced synthesis of sHsps and Hsp90 triggers *C. elegans* immunity to pathogenic *Pseudomonas aeruginosa*. Heat shock transcription factor-1 and the associated DAF2/DAF-

16 pathway are potentially involved in this protective mechanism (Singh and Aballay, 2006a), and this may be true for *Artemia* innate immunity. Hsps might also be required for cytoprotection from cell injury due to pathogen proliferation and for the proper folding of proteins synthesized in response to bacterial pathogens (Singh and Aballay, 2006b). Further studies are necessary to elucidate the relationship between Hsps and the *Artemia* immune response, with the application of molecular tools such as RNA interference (RNAi) an option (Copf et al., 2006). Whatever the outcome of such experiments, the current investigation indicates that *Artemia* can be primed against pathogenic *Vibrio* by elevating endogenous Hsp70, an intriguing finding with potential applications in aquaculture.

In related work, thermotolerance was enhanced in *Artemia* larvae exposed to combined hypo- and hyperthermic stress, an observation supporting the hypothesis that Hsps mediate thermotolerance. Similar conclusions are permitted from other results where a 1 h sub-lethal heat shock from 28 to 40°C induced Hsp70 in *Artemia* larvae and enhanced thermotolerance (Miller and McLennan, 1988a). Additionally, a temperature increase from 22 to 37°C for 30 min, followed by recovery for 1 h, induced Hsc70 and Hsp67 while generating thermotolerance in adult *Artemia* for at least 3 days (Clegg et al., 2000a). Tolerance is conferred, at least in part, because Hsps repair damaged proteins (Hightower et al., 1999; Hofmann, 1999), and protect soluble proteins against heat denaturation (Jinn et al., 1989), both vital actions in maintaining cellular homeostasis and preventing cell death.

Prompted by the emerging evidence suggesting that endogenous Hsps protect *Artemia franciscana* against pathogenic vibrios, subsequent studies examined the protective properties of exogenous Hsps. Feeding with bacteria over-producing DnaK, the prokaryotic equivalent of Hsp70, and other prokaryotic Hsps boosted *Artemia* survival approximately two- to three-fold upon challenge with pathogenic *V. campbellii*. Immunoprobing of western blots revealed that the amount of DnaK accumulating in bacteria upon induction by heat shock or by

arabinose-based stimulation of transformed cells, correlated with larval resistance to infection by *V. campbellii*. In contrast, *Artemia* fed with bacteria lacking the ability to induce Hsp expression upon heat shock were more susceptible to pathogenic vibrios, as indicated by larval mortality following *Vibrio* exposure. Exogenous Hsps, as proposed for endogenous Hsps, possibly trigger the *Artemia* innate immune response, producing anti-inflammatory substances which suppress infection. Exogenous Hsp70 administration increases inducible nitric oxide synthase and nitric oxide production (Panjwani et al., 2002; Campisi and Fleshner, 2003), as well as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 in neutrophils and macrophages (Asea et al., 2000; Campisi and Fleshner, 2003; Johnson and Fleshner, 2006). Phagocytes and granulocytes release lysozyme, reactive oxygen species, and cationic peptides upon Hsp exposure (Jacquier-Sarlin et al., 1994; Basu et al., 2002). These studies indicate that exogenous Hsps influence several immune cells, thus providing protection during pathogen challenge.

The fate of prokaryotic Hsps in *Artemia* and how this relates to immune stimulation, assuming this is the basis of increased *Vibrio* tolerance seen in this study**,** remain as interesting topics for further study. Does the resistance to vibrios shown in this study depend on Hsps reaching the *Artemia* circulatory system, as is the case for immune stimulation in other crustaceans (Lee and Söderhall, 2002)? If so, how do Hsps exit the gastrointestinal tract and enter the hemolymph, presumably to stimulate the hemocytes (Day et al., 2000). To answer these questions it will be important to localize Hsps after bacterial ingestion by *Artemia* larvae, perhaps by immunohistochemistry using antibodies against bacterial Hsp70. Moreover, Hsps are presumably degraded during digestion in the gut suggesting that sHsp peptides are sufficient to stimulate the immune system and enhance resistance against vibrios. In that respect it is interesting that an Hsp70 peptide consisting of amino acid residues 407–426 stimulates mammalian dendritic cells (Wang et al., 2005). Hence, molecular techniques

such as site-directed mutagenesis to modify Hsps (King et al., 2007) and/or over-expression of Hsp peptides may be employed to examine which part of the protein is required to induce *Vibrio* resistance through feeding.

Hsp protective effects in different developmental stages of *Artemia* and under non-gnotobiotic conditions must be investigated in order to exploit potential for disease control in *Artemia* and other commercially important fish and shrimps species. In testing these possibilities the use of sub-lethal heat shock may not be an ideal way to enhance Hsp production because acute temperature shifts are often detrimental, causing significant mortalities and adversely affecting physiological balance. Hence, less traumatic approaches are required and in this context, the regulatory effects of bio-active compounds derived from the prickly pear cactus (*Opuntia ficus indica*) which accelerate and elevate Hsps synthesis in humans (Wiese et al., 2004), may be tested. Furthermore, heat shocked algae, rotifers and other live food other than bacteria could be employed as a feed source for exogenous Hsps. Quorum-sensing molecules from *Bacillus sp.* induce Hsps production in epithelial cells (Fujiya et al., 2007). Consequently, *Bacillus* strains, often advocated as probiotic bacteria (Moriarty, 1998; Verschuere et al., 2000a), may trigger Hsp production and contribute to disease resistant in aquatic organisms. It is obvious that such an intriguing possibility needs verification.

To summarize, application of the GART system has revealed that *Artemia* resistance to *Vibrio* infection is enhanced, perhaps by stimulation of the immune response, upon elevating endogenous Hsp70 by non-lethal heat shock and through administration of heat-shocked bacteria enriched in DnaK. Both techniques represent efficient strategies to control *Vibrio* infection in *Artemia* and may serve as useful alternatives to antibiotic use in aquaculture. Elucidating the relationship between Hsps, pathogen resistance and the immune system in *Artemia* represents an intriguing challenge. With the development of new tools and

existing molecular methodologies it will be possible to dissect this complex and highly integrated relationship, revisit older questions regarding Hsp functions, and gain new insights into Hsp activity in *Artemia*.

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SUMMARY

SAMENVATTING

SUMMARY

Disease imposes an important constraint on aquaculture and in this context vibriosis brings about massive mortalities in many commercial species, ranging from fish to shrimp, with resultant heavy monetary losses. Antibiotics are frequently used to control disease but this is expensive and entails severe negative impacts on living organisms and the environment. Therefore, an urgent need exists to develop effective prophylactic measures which will reduce antibiotic use and the environmental impact of aquaculture.

During my work the application of heat shock proteins (Hsps) as an approach to disease control in aquaculture was explored, with gnotobiotic *Artemia franciscana* larvae as the model organism. In chapter 4, a non-lethal heat shock (NLHS) protocol for induction of endogenous Hsp70 in *Artemia* was optimized. Specifically, axenic larvae incubated at 28°C were heat shocked at 32, 37 and 40°C for 15, 30, 45 and 60 min, with recovery periods of 2, 6, 12 and 24 h and then tested for resistance against *Vibrio campbellii and V. proteolyticus*. An NLHS of 37°C for 30 min followed by a 6 h recovery period optimally enhanced resistance of *Artemia* larvae against pathogenic vibrios and induced Hsp70 maximally. The resulting twofold increase in survival of larvae to pathogenic *Vibrio* in concert with stress protein synthesis suggested that Hsp70 functions in protection.

The expression of Hsp70 in *Artemia* larvae following exposure to abiotic stressors including a hypothermic stress from 28°C to 4°C for 1 h, a combined hypo- and hyperthermic stress with temperature reduction from 28°C to 4°C for 1 h followed by incubation at 37°C for 30 min, and several osmotic stresses with change from 30g/l to 4, 50, 100 and 150g/l for 30 min was examined in chapter 5. The effects on animal weight loss, induced thermotolerance, and resistance against *V. campbellii* were determined. Immunoprobing of western blots revealed a single polypeptide of approximately 70 kDa, which increased only in larvae exposed to combined hypo- and hyperthermic shock. A lower ash free dry weight,

reflecting reduced growth, was detected in animals stressed at salinities of 100 and 150 g/l and from the combined hypothermic/hyperthermic treatment. Conversely, weight loss was not apparent in larvae experiencing hypothermic stress treatment and osmotic stress at 4 and 50 g/l. Thermotolerance and protection against infection by *V. campbellii* were significantly enhanced in larvae preconditioned with a combined hypo- and hyperthermic stress. The data support a causal link between Hsp accumulations as monitored by Hsp70 induced by abiotic stress and enhanced resistance to infection by *V. campbellii*, perhaps via stimulation of the *Artemia* immune system.

In chapter 6, the protective properties of exogenous Hsps were investigated. Heat shocked *E. coli* strains CAG 626 and CAG 629, and transformed *E. coli* over-expressing the three Hsp combinations DnaK-DnaJ-GrpE and GroEL-GroES, DnaK-DnaJ-GrpE, and GroEL-GroES were fed to *Artemia* larvae prior to *V. campbellii* challenge. Heat shocking strain CAG 626 enhanced its ability to safeguard *Artemia* larvae from *Vibrio* infection by approximately twofold whereas CAG 629, which does not increase Hsp production upon heat shock, failed to provide protection. Feeding *Artemia* larvae with *E. coli* over-producing DnaK-DnaJ-GrpE significantly increased larval resistance to *V. campbellii*, suggesting a role for these proteins in protection, possibly via immune enhancement. In contrast, this effect was not observed in larvae fed with *E. coli* over-producing GroEL-GroES. To test the role of DnaK larvae were fed with *E. coli* over-producing only this protein and this led to a 3-fold increase in *Artemia* survival.

The protective effects of bacterially encapsulated heat shock proteins in *Artemia* larvae were investigated further in chapter 7. Heated bacterial strains LVS 2 (*Bacillus sp*), LVS 3 (*Aeromonas hydrophila*), LVS 8 (*Vibrio sp*), GR 8 (*Cytophaga sp*) and GR 10 (*Roseobacter sp*), verified by probing of western blots to over-produce DnaK, were fed once to gnotobiotic *Artemia* larvae and their effects were examined in *V. campbellii* challenges.

All heated bacterial strains were more effective than non-heated bacteria in defending gnotobiotic *Artemia* larvae against *V. campbellii* challenge. Immunoprobing of western blots and quantification by ELISA revealed that the amount of DnaK in bacteria, and their ability to enhance larval resistance to *V. campbellii* infection, were correlated. DnaK and/or other Hsps, improved resistance of *Artemia* against *Vibrio* infection, perhaps by immune stimulation.

In conclusion, the outcome from this PhD work revealed that *Artemia* resistance to *Vibrio* infection is enhanced, perhaps by stimulation of the immune response, upon elevating endogenous Hsps by non-lethal heat shock and through administration of heat-shocked bacteria. The feeding of *E. coli* under an arabinose inducible promoter, also providing protection against *V. campbellii*, clearly placed Hsp70 as the focal point of attention. Both techniques represent efficient strategies to control *Vibrio* infection in *Artemia* and may serve as alternatives to antibiotic use in aquaculture.

SAMENVATTING

Ziekten zijn één van de voornaamste belemmeringen voor de groei van de aquacultuur. In deze context veroorzaakt vibriosis massale sterfte in verschillende gekweekte species, gaande van vissen tot garnaalachtigen, met zware economische verliezen als gevolg. Antibiotica worden frequent gebruikt om ziekten te behandelen, maar dergelijke behandelingen zijn duur en gaan gepaard met belangrijke negatieve invloeden op levende organismen en op het milieu. Als gevolg daarvan is er een dringende noodzaak om effectieve profylactische behandelingen te ontwikkelen om het antibioticumgebruik en de daarmee gepaard gaande negatieve impact van de aquacultuur op het milieu te reduceren.

In dit werk werd het toepassen van hitteschokproteïnen (Hsps) onderzocht als een mogelijke manier om infecties te bestrijden in de aquacultuur. Daarbij werden gnotobiotische *Artemia franciscana* larven als modelorganismen gebruikt. In *Chapter 4* werd een protocol geoptimaliseerd om de endogene aanmaak van Hsp70 in *Artemia* te induceren, gebruik makend van een niet-lethale hittebehandeling. Daartoe werden axenische larven, die bij 28°C geïncubeerd werden, gedurende 15, 30, 45 en 60 min bij 32, 37 en 40°C geplaatst. Vervolgens werden ze na een recuperatieperiode van 2, 6, 12 of 24 u, getest voor resistentie tegen infecties met *Vibrio campbellii* en *Vibrio proteolyticus*. Een hittebehandeling bij 37°C gedurende 30 min, gevolgd door een recuperatieperiode van 6u resulteerde in een maximale aanmaak van Hsp70 en gaf de grootste resistentie van *Artemia* larven tegen de pathogene vibrios. De resulterende tweevoudige verhoging in overleving van de larven, samen met de synthese van stress eiwitten, suggereert dat Hsp gemeten via Hsp70 een rol speelt bij bescherming tegen infecties.

De expressie van Hsp70 in *Artemia* larven na blootstelling aan abiotische stress factoren, waaronder een hypotherme stress (van 28°C naar 4°C gedurende 1 u), een gecombineerde hypo- en hypertherme stress (tijdens 1 u van 28°C naar 4°C, gevolgd door

een incubatie bij 37°C gedurende 30 min) en verschillende osmotische stressbehandelingen (van 30 g/l zout naar 4, 50, 100 en 150 g/l gedurende 30 min) werden onderzocht in *Chapter 5*. Er werd nagegaan wat de invloed is van deze stress factoren op gewichtsverlies, geïnduceerde thermotolerantie en resistentie tegen *V. campbellii* bij de larven. Western blots met immunodetectie wees op één enkel polypeptide van ongeveer 70 kDa, dat enkel in verhoogde mate werd aangemaakt in larven die blootgesteld werden aan de gecombineerde hypo- en hypertherme behandeling. Een lager asvrij droog gewicht (wijzend op vertraagde groei) werd opgemerkt in dieren die behandeld werden bij zoutgehaltes van 100 en 150 g/l en bij de gecombineerde hypotherme/hypertherme behandeling. Daarentegen werd geen gewichtsverlies waargenomen in larven die blootgesteld werden aan hypotherme stress en osmotische stress bij 4 en 50 g/l zout. Thermotolerantie en bescherming tegen infecties met *V. campbellii* waren significant hoger in larven die voorbehandeld waren met een gecombineerde hypo- en hypertherme stress. Deze data bevestigen een causaal verband tussen de accumulatie van Hsp (bvb Hsp70) na blootstelling aan abiotische stress en een verhoogde resistentie tegen infectie met *V. campbellii*, mogelijk door de stimulatie van het afweersysteem van *Artemia*.

In *Chapter 6* werden de beschermende eigenschappen van exogene Hsps onderzocht. Hittebehandelde *E. coli* stammen CAG 626 en CAG 629 en getransformeerde *E. coli* die de drie Hsp combinaties DnaK-DnaJ-GrpE en GroEL-GroES, DnaK-DnaJ-GrpE, en GroEL-GroES tot overexpressie bracht, werden gevoederd aan *Artemia* larven vóór blootstelling aan *V. campbellii*. Hittebehandeling van stam CAG 626 verhoogde zijn vermogen om *Artemia* larven tegen infectie met de *Vibrio* te beschermen ongeveer twee keer, terwijl stam CAG 629, die Hsp niet verhoogd tot expressie brengt bij hittebehandeling, geen bescherming kon bieden. Het voederen van *Artemia* larven met *E. coli* die DnaK-DnaJ-GrpE overproduceert, zorgde voor een significant verhoogde resistentie tegen *V. campbellii*, wat suggereert dat deze

eiwitten een rol spelen bij de bescherming, mogelijk via stimulatie van het afweersysteem. Een dergelijk effect werd daarentegen niet waargenomen in larven die gevoederd waren met de *E. coli* die GroEL-GroES overproduceert. Om de invloed van DnaK te onderzoeken, werden tenslotte larven gevoederd met *E. coli* die enkel dit eiwit tot overexpressie brengt en dit zorgde voor een drievoudige verhoging in de overleving van *Artemia*.

De beschermende effecten van in bacteriën geïncapsuleerde *heat shock* eiwitten, werden verder onderzocht in *Chapter 7*. Hittebehandelde LVS2 (*Bacillus sp*), LVS3 (*Aeromonas hydrophila*), LVS8 (*Vibrio sp*), GR8 (*Cytophaga sp*) en GR10 (*Roseobacter sp*), waarvan de productie van DnaK werd bevestigd met Western blots, werden éénmalig gevoederd aan gnotobiotische *Artemia* larven en hun invloed werd bestudeerd bij blootstelling aan *V. campbellii*. Alle hittebehandelde stammen boden de *Artemia* larven een betere bescherming tegen *V. campbellii* dan niet-behandelde bacteriën. Western blots met immunodetectie en quantificatie met ELISA toonde aan dat de hoeveelheid DnaK in de bacteriën positief gecorreleerd was met hun vermogen om de larven te beschermen tegen *V. campbellii*.

Tot conclusie, de resultaten van dit doctoraatsonderzoek tonen aan dat de resistentie van *Artemia* tegen infecties met vibrio's verbeterd wordt door het verhogen van het gehalte aan endogene Hsp (bvb Hsp70) (verkregen door een niet-lethale hittebehandeling op de larven) en door het toedienen van hittebehandelde bacteriën (die door de behandeling aangerijkt waren aan DnaK), vermoedelijk door de stimulatie van de afweerrespons. Het vervoederen van *E. coli* cellen die DnaK overproduceren onder een arabinose induceerbare promotor, wat ook leidt tot bescherming tegen *V. campbellii*, plaatst Hsp70-type van moleculen in het focale punt van de belangstelling. Beide technieken zijn efficiënt om *Vibrio* infecties te bestrijden in *Artemia* en zouden mogelijk een nuttig alternatief kunnen zijn voor antibiotica in de aquacultuur.

CURRICULUM

VITAE

Curriculum Vitae

1. Personal Information

Name	:	Yeong Yik Sung
Gender	:	Male
Nationality	:	Malaysian
Date of Birth	:	13 May 1977
Place of Birth	:	Penang, Malaysia
Race	:	Chinese
Language Spoken	:	Malay, English, Chinese (Mandarine, Cantonese, Fujian)

2. Academic Qualifications

1997 – 2000 : B. Sc. Degree in Fisheries Science, Faculty of Science and Technology, Universiti Putra Malaysia

Major subjects: Aquaculture, Ornamental fish culture

2001 – 2003 : M. Sc. (Biotechnology), Faculty of Agrotechnology and Food Science, Kolej Universiti Sains dan Teknologi Malaysia (KUSTEM)

Major subjects: Biotechnology, Aquaculture, Ornamental fish culture

2004 – 2008 : Doctoral training in Applied Biological Sciences. Faculty of Bioscience Engineering, Ghent University, Belgium.

Major subjects: Live food culture, Heat shock proteins, Immunology

3. Relevant working experience

February 2001: Faculty of Science and Technology, University Putra Malaysia Terengganu, Malaysia

Research assistant
Integrated in a scientific project relating to "Broodstock management and larval rearing of Gouramis" (IRPA project) from the grant funded by the Ministry of Science, Technology and the Environment (MOSTE) coordinated by Dr. Abol Munafi Ambok Bolong.

October 2001: Faculty of Science and Technology, University Putra Malaysia Terengganu, Malaysia

Demonstrator
Integrated in the laboratory practicum of the subjects "Fundamentals of Microbiology" and "Applied Microbiology" coordinated by Associate Prof. Dr. Guruprasad A. Sulebele.

2003 - 2004: Faculty of Agrotechnology and Food Science, University College of Science and Technology Malaysia (KUSTEM)

Tutor
Integrated in the teaching of the subject "Ornamental fish culture" coordinated by Dr. Abol Munafi Ambok Bolong.

2004 onwards: Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Belgium.

Research scientist
Integrated in the scientific projects "Functional role and characteristics of microorganisms in the larviculture of aquatic organisms: *Artemia* as preferred test organism" (no. 350230.02) and the project "Nutritional and immunostimulatory characteristics of isogenic yeast mutants in *Artemia*" (no. 1.5.125.04) funded by the Belgian Foundation for Scientific Research (FWO). Scientific coordinators: Prof. Dr. ir. Peter Bossier and Prof. Dr. Patrick Sorgeloos.

4. Awards and scholarships

Cargill Prize (2000) – Award for "Outstanding Thesis" in Fisheries and Aquaculture category in University Putra Malaysia.

National Science Fellowship (2001 - 2003) - Award of scholarship by the Ministry of Science, Technology and the Environment, Malaysia (MOSTE) for M.Sc. (Biotechnology) study in KUSTEM.

KUSTEM/UMT Scholarship (2004 – 2008) - Award of scholarship by University Malaysia Terengganu (formerly known as Kolej Universiti Sains dan Teknologi Malaysia) for Ph.D study in Ghent University, Belgium.

5. Thesis supervision

2006-2007 (Co-promotor)

Carlos Pineda Sánchez-Garrido – Effect of abiotic stress on Hsp-70 expression and cross-tolerance of *Artemia franciscana* nauplii against pathogenic *Vibrio campbellii*. Master of Sciences in Aquaculture at the Faculty of Agricultural and Applied Biological Sciences, Ghent University, Belgium. p. 55.

Chen Shuaijun – A study of bacterial *E. coli* encapsulated Hsp-70 as a dietary feed for the enhancement of immune capability of gnotobiotically grown *Artemia franciscana* larvae. Master of Science in Aquaculture at the Faculty of Agricultural and Applied Biological Sciences, Ghent University, Belgium. p. 48.

Tran Nguyen Hai Nam – Larval performance of *Artemia* fed bacteria encapsulated Hsps in open culture system. Master of Science in Aquaculture at the Faculty of Agricultural and Applied Biological Sciences, Ghent University, Belgium. p. 52.

2005-2006 (Co-promotor)

Martha Fikru Ashame - A study of the immunostimulatory effects of gnotobiotically grown *Artemia franciscana* by using *Escherichia coli* as a vector of heat shock proteins. Master of Science in Aquaculture at the Faculty of Agricultural and Applied Biological Sciences, Ghent University, Belgium. p. 64.

6. Publications

Scientific papers in SCI peer-reviewed journals

Yeong, Y. S., Van Damme, E. J. M., Sorgeloos, P. and Bossier, P. (2007). Non-lethal heat shock protects gnotobiotic *Artemia franciscana* larvae against virulent Vibrios. *Fish Shellfish Immunol* **22,** 318-326. (IF: 2.725)

Yeong, Y. S., Sánchez-Garrido, C. P., MacRae, T. H., Sorgeloos, P. and Bossier, P. (In Press). Exposure of gnotobiotic *Artemia franciscana* larvae to abiotic stress promotes heat shock protein 70 synthesis and enhances resistance to pathogenic *Vibrio campbellii*. *Cell Stress Chaperones.* (IF: 3.097)

Yeong, Y. S., Ashame, M. F., Chen, S. J., MacRae, T. H., Sorgeloos, P. and Bossier, P. DnaK, the 70-kDa bacterial heat shock protein, protects *Artemia* larvae from *Vibrio* infection. (Submitted).

Papers presented in national and international conference

Oral presentation

Yeong, Y. S., Pineda, C., MacRae, T. H., Sorgeloos, P. & Bossier, P. Effects of abiotic stress on Hsp-70 expression and cross-protection of gnotobiotic *Artemia franciscana* nauplii against pathogenic *Vibrio campbellii.* Paper presented at the Asian-Pacific Aquaculture Conference 2007 from 5-8, August 2007 in Hanoi, Vietnam.

Yeong, Y. S., Abol-Munafi, A. B, Hasan, S. M. Z. and Shazali, N. A. M. Studies on the genetic variation of Gourami (*Trichogaster* sp.) using PCR-RAPD techniques. Paper presented at the 2nd National Fisheries Symposium from 18-20, February 2003 in Kelantan, Malaysia.

Yeong, Y. S., Abol-Munafi, A. B., Ang K. H., Shazili, N. A. M., and Hasan S. M. Z. A study on the first feeding of gourami (*Trichogaster spp.*) larvae by using different artificial diets and probiotic bacteria combinations. Paper presented at the Malaysian Science and Technology Congress 2002 from 12-14, December 2002 in Kuching, Malaysia.

Poster presentation

Yeong, Y. S., Dhont, J. Sorgeloos P. and Bossier P. Non-lethal heat shock protects *Artemia franciscana* against virulent Vibrios. Poster presented at the 4th Fish & Shellfish Larviculture Symposium from 5-8, September 2005 in Ghent, Belgium.

Yeong, Y. S., Abol-Munafi, A. B., Shazili, N. A. M., and Hasan S. M. Z. Hatching, survival and growth of gouramis (*Trichogaster spp.*) in different rearing media. Poster presented at the Malaysian Science and Technology Congress 2002, from 12-14, December 2002 in Kuching, Malaysia.

Yeong, Y. S., Abol-Munafi, A. B., Ang, K. H and Shazili, N. A. M. A study on the first feeding of Pearl Gourami (*T leeri*) and Three Spot Gourami (*T. trichopterus*) larvae by using different artificial diets and probiotic bacteria combinations. Poster presented at the World Aquaculture Conference 2002 from 23-27, April 2002 in Beijing, China.

A. B. Abol-Munafi, **Yeong, Y. S.** and Shazili, N. A. M. Hatching, survival and growth of Pearl Gourami (*T.leeri*) and Three Spot Gourami (*T. trichopterus*) in different rearing media. Poster presented at the World Aquaculture Conference 2002 from 23-27, April 2002 in Beijing, China.

Yeong Y. S., A. B. Abol-Munafi, and Shazili, N. A. M. Effect of calcium (Ca^{2+}) on hatching, survival, growth and scales formation of Pearlscale goldfish. Poster presented at World Aquaculture Conference 2002 from 23-27, April 2002 in Beijing, China.

Abol-Munafi, A. B., **Yeong, Y. S.** and Shazili, N. A. M. Effects of calcium (Ca^{2+}) on hatching rate, survival rate, larval abnormalities and growth rate of Pearlscale goldfish larvae (*Carassius auratus*). Poster presented at the National Fisheries Symposium from 31 Oct –2 Nov, 2000 in Johor Bahru, Malaysia.

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Yeong Yik Sung