Heat Shock Proteins 12
Series Editors: Alexzander A. A. Asea · Stuart K. Calderwood

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Heat Shock Proteins in Veterinary Medicine and Sciences

Springer

Heat Shock Proteins

Volume 12

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Heat Shock Proteins: key mediators of Health and Disease. Heat shock proteins (HSP) are essential molecules conserved through cellular evolution required for cells to survive the stresses encountered in the environment and in the tissues of the developing and aging organism. These proteins play the essential roles in stress of preventing the initiation of programmed cell death and repairing damage to the proteome permitting resumption of normal metabolism. Loss of the HSP is lethal either in the short-term in cases of acute stress or in the long-term when exposure to stress is chronic. Cells appear to walk a fine line in terms of HSP expression. If expression falls below a certain level, cells become sensitive to oxidative damage that influences aging and protein aggregation disease. If HSP levels rise above the normal range, inflammatory and oncogenic changes occur. It is becoming clear that HSP are emerging as remarkably versatile mediators of health and disease. The aim of this series of volumes is to examine how HSP regulation and expression become altered in pathological states and how this may be remedied by pharmacological and other interventions.

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Preface

Veterinary medicine is designed to advance our understanding and to promote innovative advances in basic and clinical veterinary sciences with the goal of improving the health and well-being of animals and, through them, the health, stability, and economic development of humans.

The book *Heat Shock Proteins in Veterinary Medicine and Sciences* provides the most comprehensive review on contemporary knowledge on the role of heat shock proteins (HSP) in veterinary medicine and sciences. Using an integrative approach to understanding heat shock protein physiology, the contributors provide a synopsis of novel mechanisms by which HSP is involved in the regulation of normal physiological and pathophysiological conditions.

To enhance the ease of reading and comprehension, this book has been subdivided into various sections: Section I reviews current progress on the role of HSP in relation to physiology and diseases in domestic animals, Section II evaluates the role of HSP as it relates to antioxidant and thermal stress responses in poultry, Section III focuses the reader on the role of heat shock proteins in aquatic animals, Section IV concentrates the reader's attention on the role of HSP in disease-causing parasites that plague animals.

Key basic and clinical research laboratories from major universities and veterinary hospitals around the world contribute chapters that review present research activity and importantly project the field into the future. The book is a must-read for veterinary doctors, researchers, postdoctoral fellows, and graduate students in the fields of veterinary medicine, animal physiology, animal husbandry, biotechnology, molecular medicine, microbiology, and pathology.

Toledo, Ohio, USA — Alexzander A. A. Asea
Houston, Texas, USA — Punit Kaur

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Editors Biography

Prof. Dr. Alexzander A. A. Asea is a highly innovative and accomplished world-renowned clinical and basic research scientist and visionary executive leader who has exceptional experience spearheading clinical and basic science research, training, education, and commercialization initiatives within top ranked academic biomedical institutes. Prof. Asea's initial findings studying the effects of Hsp72 on human monocytes lead to the proposal of a novel paradigm that Hsp72, previously known to be an intracellular molecular chaperones, can be found in the extracellular milieu where it has regulatory effects on immunocompetent cells – a term now called chaperokine. Prof. Asea has authored over 255 scientific publications including peer-reviewed articles, reviews, books, book chapters, editorials, and news headliners in a wide range of biomedical-related disciplines. Prof. Asea is the series editor of the widely successful book series *Heat Shock Proteins* (Springer Nature Publications) and is an editorial board member of 13 other scientific peer-reviewed journals. Currently, Prof. Asea is at the University of Toledo College of Medicine and Life Sciences in Toledo, USA.

Dr. Punit Kaur is an expert in onco-proteogenomics, with extensive training and experience in quantitative mass spectrometry imaging, protein chemistry, and biomarker discovery. Dr. Kaur's main research focus is on the use of heat-induced nanotechnology in combination with radiotherapy and chemotherapy in the cancer stem cell therapy. Dr. Kaur has published more than 40 scientific articles, book chapters, and reviews, and currently serves as editorial board member for the *European Journal of Cancer Prevention and the Journal of Proteomics and Bioinformatics*. Dr. Kaur is an editor of five books in the highly successful *Heat Shock Proteins* book series by Springer Nature Publishers. Currently, Dr. Kaur is a Visiting Scientist Professor at the University of Texas MD Anderson Cancer Center in Houston, USA.

Part I
Domestic Animals

Chapter 1
Thermotolerance in Domestic Ruminants: A HSP70 Perspective

Iqbal Hyder, Manjari Pasumarti, Poonooru Ravikanth Reddy, Chigurupati Srinivasa Prasad, Kamisetty Aswani Kumar, and Veerasamy Sejian

Abstract Thermal stress is one of the most important factors limiting ruminant production and thermotolerance studies in domestic ruminants has lot of bearing on the identification of prospective biomarkers for thermal stress, especially the heat stress. Heat stress in ruminants is characterized by heat shock response, which is mediated by different types of Heat Shock Proteins (HSP) like HSP60, 70, 90, 110, 27 among which some play a critical role in the initial stages of heat stress and some in the later stages. Among all HSP, HSP70 is considered as cellular thermometer and is indicator of quantum of stress experienced by the cell. At a given amount of stress the expression of HSP70 varies with species, breed, age and type of tissue indicating the variations in thermotolerance. Members of HSP70 family have many homologues like HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA4, HSPA5, HSPA6 & HSPA8 of which some are constitutive and some are inducible. These genes are elevated with heat stress in different type of cells at variable rate. The differences in thermotolerance among species and breeds are correlated with variations in different HSP70 family members. HSP70 can be viewed as prospective biomarker for marker assisted selection in animals in order to have more thermotolerant animals in future as a strategy towards Climate resilient ruminant production.

Keywords Adaptability · Heat Shock Proteins · HSP70 · Ruminants · Thermal stress · Thermotolerance

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Abbreviations

ACTH	Adrenocorticotropic hormone
ARD	Average relative deviation
CCI	Comprehensive climate index
CRH	*C*orticotropin releasing hormone
FAO	Food and Agriculture organization
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPA	Hypothalamo-pituitary-adrenal
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
IL	Interleukin
IPCC	Intergovernmental Panel on Climate Change
LCT	Lower critical temperature
MEC	Mammary epithelial cells
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
RAD	Radia*tion*
RH	Relative humidity
RR	Respiration rate
RT	Rectal temperature
SNP	Single nucleotide polymorphism
TDI	Tuncia Dartos index
THI	Temperature humidity index
TI	Thermal index
TNF	Tumor necrosis factor
TNZ	Thermoneutral zone
UCT	Upper critical temperature
UTR	Untranslated region;
WS	Wind speed

1.1 Introduction

The animal agriculture has played a crucial role in the evolution of human civilizations, since domesticated species are renewable sources that provide humans with food and other tangible, intangible benefits. Especially the ruminants like cattle, sheep, goat and buffalo have always been a part and parcel of human habitations which is evident from various sources of human history. The ruminants over a period of time got adapted to thrive in different parts of the planet emerging as the most successful herbivores among mammals represented by 200 species among

which there are 75 million wild and 3.5 billion domesticated animals across the globe (Hackman and Spain 2010) and some of them can survive in areas of feed/fodder shortage even up to less than half of their dietary requirement (Hyder et al. 2013). Today, livestock production is a dynamic and integral part of the food system, contributing 40% of the global value of agricultural output, 15% of total food energy, and 25% of dietary protein and supporting the livelihoods and food security of almost a billion people. The exploding population, globalization driven urbanization coupled with rising incomes during the last three decades have created a demanding situation on livestock products particularly in developing countries (FAO 2009). The World Bank estimates predicted that meat production should increase by 80% by 2030 that calls for efficient use of available animal resources. At the same time, the ruminant production is mired in several challenges with climatic factors like temperature, humidity, solar radiation etc. recognized as potential hazards in growth and production of livestock (Ganaie et al. 2013). Thermal stress seems to be exerting drastic effects on animal production that includes both heat and cold stress with the former being most important in major parts of ecosystems where ruminant domestication is prevalent. Heat stress forces a significant financial burden on livestock producers by decreasing production, reproductive efficiency and adversely affecting livestock health (Hyder et al. 2017). Increasing frequencies of heat stress are estimated to be more likely as a result of climate change and these will have adverse effects on livestock productivity (IPCC 2007). It is being predicted that these effects are going to be much more aggravated as there is a global consensus in the scientific community that climate change is already happening and that the further change is inevitable since by the year 2100 global temperatures may raise by 3.7–4.8 °C (IPCC 2014; Quere et al. 2014). Heat stress is a result of unfavorable negative balance between the net amount of energy flowing from the animal to its surrounding environment and the amount of heat energy produced by the animal (Hyder et al. 2017). Apart from the production aspects, in recent years, there is growing awareness regarding welfare of animals and heat stress is one of the major detrimental factors for animal welfare. The concern with the thermal comfort of livestock is justifiable not only for tropical countries that are characterized by high temperature and humidity, but also for temperate zone nations in which high ambient temperatures are becoming an issue (Nardone et al. 2010). It is expected that 20–30% of livestock will be at the risk of extinction (FAO 2007) due to changes in climate. There is evidence that climate change, especially elevated temperature has already changed the overall abundance, seasonality and spatial spread of farmed small ruminants (Van Dijk et al. 2010).

Hence, considering the multitude of effects due to thermal stress in animals, several adaptation measures have been recommended by the scientist across the globe and there is a consensus that it is generally faster to improve welfare, production and reproduction performances by altering the environment (Mader et al. 2006). But given the financial constraints of tropical farmers involved in ruminant production, intense environmental modifications like air conditioning, designer buildings etc. may be too expensive and economically unviable. This warrants for animals with improved thermotolerance that can also possess fair

production and reproduction abilities so that an economic breakeven can be attained (Collier et al. 2005). Therefore, proper breed selection is a very valuable tool for sustaining animal production under an increasingly challenging environment.

The intended selection of thermotolerant animals/breeds will be successful if we have suitable biomarkers that can be used to select the animals and thereby breed them to establish heat resilient herds, one of the much sought aspects for climate resilient animal agriculture. Thermotolerance is a biological response which enables organisms to survive sub lethal high temperatures prior to experiencing a non-lethal heat exposure (Pawar et al. 2014). Several studies proved that Heat shock proteins (HSP) form a primary system for intracellular self-defense which are necessary for the cellular homeostasis especially important in a stressful environment. Among all the HSP, HSP70 is of particular interest since it is visualized as a rescue marker for cells that are vulnerable for stress induced cellular damage. With this background, the present chapter is targeted to enlighten the readers about the various aspects of thermotolerance in domestic ruminants from the purview of HSP70. Effort has been made to collect, arrange and synthesize information, deduce suitable and possible corollaries pertaining to alterations in HSP70 during various types of thermally induced stress conditions. For better comprehension of the heterogenous group of readers, the brief background about heat stress, indices to measure the heat stress, adaptation strategies of ruminants to heat stress are also presented in this chapter. Though thermal stress encompasses both heat and cold stress, majority of the chapter is emphasized on the former than latter since heat stress is more common and harmful for ruminant production due to spatial distribution of most of the domestic ruminants in the tropical areas with some salient aspects covered on the cold stress aspects too.

1.1.1 Thermal Stress as the Important Factor Influencing Livestock Production

Stress has been defined by various scientists taking into account multiple contexts. Some define it as a body reaction to stimuli that alters normal physiological equilibrium or homeostasis, often with detrimental effects (David et al. 1990) whereas some others view it as cumulative detrimental effect of a variety of factors on the health and performance of animals, or the magnitude of forces external to the body that tend to displace its systems from its basal state (Silanikove 2000a). Stress can also be defined as the inability of an animal to cope up with its environment that is reflected in failure of reaching genetic potential (Dobson and Smith 2000). In general, the homeothermic animals have a range of temperature where they do not have to expend energy in order to maintain their core body temperature, which means most of the energy can be diverted for production. This is called thermoneutral zone (TNZ) that is normally in the scale of 4 and 25 °C for most of the farm animals and the temperatures exceeding 25 °C will result in Heat stress (Mishra and Palai 2014; Dangi et al. 2015). When the ambient temperature on either side

of the scale exceed the limits, it results in a condition where the energy reserves of the animal are diverted for maintenance of homeostasis at the cost of production, resulting in so called thermal stress. The thermal stress due to temperature going below the lower limit of the TNZ i.e., "lower critical temperature (LCT)", is termed as cold stress and when it goes above the limit of TNZ i.e., the "upper critical temperature (UCT)" is termed as heat stress. Generally, the TNZ of an animal depends on age, species, breed, feed intake, diet composition, previous state of temperature, acclimation or acclimatization, production, specific housing and pen conditions, tissue insulation, external insulation, and behavior of an animal (Yousef 1985). For example, the TNZ of goats at 60–70% relative humidity, 5–8 km/h wind velocity and a medium level of solar radiation is 13–27 °C (Misra and Puneet 2009). In addition, genetic improvement programs aimed at enhancing production traits could well increase an animal's susceptibility to high environmental temperatures due to the close relationship between metabolic heat generation and production level (Kadzere et al. 2002) making it as one of the important factors determining TNZ.

Heat Stress is described as the perceived discomfort and physiological strain associated with an exposure to an extreme and hot environment (Gupta et al. 2013). Though ambient temperature is considered as an important parameter to be regulated for optimal ruminant production, the animals actually experience what is called as "effective temperature" which is determined by other meteorological variables along with ambient air temperature. The most commonly used measure to assess this effective temperature especially at higher temperatures is Temperature Humidity Index (THI) (Thom 1959) and for colder climates, it is Wind Chill Index (Siple and Passel 1945). Apart from these two indices, others like Black Globe-Humidity Index (Buffington et al. 1981), Effective Temperature for dairy cows (Yamamoto 1983), Equivalent Temperature Index for dairy cows (Baeta et al. 1987), Thermal Comfort Index for sheep (Da Silva and Barbosa 1993), Heat Load Index for beef cattle (Gaughan et al. 2002), and Environmental Stress Index (Moran et al. 2001) also exist. As indices for heat and cold stress are separate, a comprehensive climate index (CCI) was proposed by Mader et al. (2010) which incorporates adjustments for Relative Humidity, Wind speed, and Radiation over conditions that encompass both cold and hot environmental conditions. In spite of THI being most widespread indicator of heat stress, its application is limited since it is an empirical representation that assumes all animals react similarly to environmental stressors, without accounting for other environmental effects (e.g., WS and RAD) and cow-specific effects (e.g., age and breed). New thermal indices (TI) that incorporated those environmental and physiological data in addition to cow specificities (e.g., breed and age) have been developed to overcome the various THI limitations (Gaughan et al. 2008; Mader et al. 2010). Several indices to measure heat tolerance have been developed over the years involving the biological factors. The THI was adjusted for wind and solar radiation based on changes in panting scores (Mader et al. 2006) and on a respiration rate index using dry bulb temperature, relative humidity (RH), wind speed (WS), and solar radiation (Eigenberg et al. 2005). Marai et al. (2007) suggests the use of average relative deviations (ARD) from normal (positive or negative) in

Table 1.1 Impact of heat stress on the ruminant production parameters

Growth	Meat, Milk & Fiber Production	Reproduction	Distribution of livestock diseases
Reduced body weight	Reduction in meat, milk, wool and hair production	Reduced fertility rate	Altered patterns of diseases in animals
Reduced average growth rate	Changes in the composition of milk	Reduced intensity, duration of estrus	Emergence of new diseases
Decrease in body condition scoring	Increase in pH of meat post slaughter	Decreased quality of oocytes	Change in the prevalence of existing diseases
Reduced birth weight	Reduction in protein content of meat	Reduced estradiol, progesterone, LH level	Changes in distribution and the abundance of disease vectors
	Reduction in marbling percentage of meat	Increased embryonic mortality	

thermal, water and/or nitrogen balances of the animals (or in all traits measured) due to exposure to hot climates for detection of adaptability to a hot climate. Other indices such as tunica dartos indices (TDI; Marai et al. 2006) have been used, as during high ambient temperatures, the tunica dartos muscle extends to dissipate as much of the excess heat as possible from the testes.

Heat Stress has emerged as one of the grave concerns for sustainable livestock production in the context of ever-changing climatic scenario (Silanikove and Koluman 2015), especially in the tropical, subtropical, arid and semiarid regions of the world (Marai et al. 2007; Nardone et al. 2010; Al-Dawood 2015). Though the heat stress markedly affects ruminants by directly reducing their production, it is also having potential to exert the effects encompassing generations. For example, heat stress was found to result in fetal malnutrition, and eventually fetal growth retardation (Tao and Dahl 2013). In fact, Tao et al. (2012) reported that cooling cows during the dry period increased the birth weight of their calves with their colostrums bearing higher IgG levels as compared to those that were not cooled. Table 1.1 summarizes the various impacts of heat stress on livestock productive performances.

The effects on animal production due to heat stress can be attributed to cellular functional alterations (Lindquist 1986; Kuhl and Rensing 2000) that include inhibition of DNA replication, transcription, translation; denaturation, misaggregation and degradation of proteins; alterations in metabolism that lead to a net reduction in cellular ATP; and membrane associated changes. Whereas the cold stress can also produce alterations in the properties of the lipid bilayer, some of which (such as phase transitions) are simply due to the reduction in temperature, others (such as changes in the fatty acid composition of the membrane) likely reflect a cellular, physiological response to cold stress. Depending on the intensity of the exposure, cold stress can trigger a cell stress response, activate the apoptotic program, or lead to necrosis (Sonna et al. 2002).

1.1.2 Mechanisms Involved in Thermotolerance of Ruminants

Thermal adaptability is a complex phenomenon characterized by involvement of various organ systems and their metabolic functions in temperature induced stress conditions that enable the animal to successfully survive and maintain optimum productivity (Shearer and Beede 1990). This adaptability levels are varied, not only between species, but also between breeds and even between individuals within breed.

The thermotolerance is achieved by various measurable changes like physiological, behavioral on a macro scale and cellular, molecular on a micro scale. Physiological Adaptations are internal systematic responses to external stimuli in order to help an organism maintain homeostasis. The cardinal physiological responses i.e., respiration rate, pulse rate, rectal temperature and sweating rate vary with the changes in season in an effort to maintain normal body temperature independent of the fluctuation in ambient temperature. Rectal temperature (RT) and respiration rate (RR) are the most sensitive indices of heat tolerance among the physiological reactions studied (Verma et al. 2000). Increased respiration rate is the first reaction when animals are exposed to environmental temperatures above the thermo-neutral zone that helps in thermolysis and heat dissipation by evaporative cooling (Maurya et al. 2007; McManus et al. 2009). In addition, exposure to heat stress also increases rectal temperature and heart rate (Al-Dawood 2017). Similarly, in ruminants where there is inefficient heat dissipation during high temperatures, body temperature rises (Silanikove 2000b; Mader et al. 2006). Sweating rate is yet another important phenomenon to lose heat by the principle of latent heat of vaporization and Muller (1982) showed that higher sweat gland volume is related to higher sweat production that confers better adaptation to tropical climates.

Apart from the physiological alterations, ethological responses driven by internal milieu can also play a significant role in animal adaptation to fluctuations in ambient temperature. Though, these behavioural changes seem voluntary, they are directed by internal drives of the animal resulting from certain changes in internal milieu. Some of them include shade seeking, grazing around the water bodies in extensive system of farming and deliberate contact with cool floors and intermittent feeding etc. in intensive system of farming.

Neuroendocrine responses play a critical role in stress and are of utmost importance in maintenance of homeostasis in livestock. Extensive studies have been done with regard to stress circuitry and its inhibitory role on growth and production (Sejian 2013; Sejian et al. 2013). Substantial evidence suggests that neuroendocrine responses vary with the type of stressor that are specific and graded, rather than 'all or none'. While acute responses have important adaptive functions and are vital to coping and survival, chronic stressors elicit endocrine responses that may actually contribute to morbidity and mortality (Sejian et al. 2010).

Activation of the stress axis is accomplished through the release of several neurotransmitters and hormones. The stress axis or the hypothalamo-pituitary-adrenal (HPA) axis consists of 3 components: corticotrophin releasing hormone or corticoliberin (CRH) neurons in the hypothalamus, corticotrophs in the anterior pituitary

and the adrenal cortex. A variety of molecular mediators have been implicated in the stimulation of CRH neurons ranging from neurotransmitters such as catecholamines to pro-inflammatory cytokines. CRH is an obligatory and primary stimulus for adrenocorticotropin hormone (ACTH) secretion by the pituitary gland. Subsequently, ACTH stimulates glucocorticoid synthesis from the cells of adrenal cortex. Glucocorticoid is the final activation product in the HPA axis and the primary effector molecule of this neuroendocrine circuit (Sejian 2013). Hence, the thermal stress response is predominantly a vertical axis response with many horizontal modulating forces.

1.1.3 Components of Heat Shock Response in Livestock

An outcome of multiple studies on thermal stress in livestock revealed that heat stress is regulated in two stages i.e., short term and long term (Garret et al. 2009). Whereas the former includes the heat shock response at the cellular level, the latter results in acclimation to stressor, involving reprogrammed gene expression and metabolism (Horowitz 2002; Collier et al. 2006). Heat acclimation is a biological adaptation to reduce physiological strain (McClung et al. 2008) whereas the heat shock response is a rapidly induced phenomenon that develops post acute heat stress though it takes about 24 h to observe the changes (Maloyan et al. 1999). Discovery of the so called heat shock response was first done in *Drosophila*, where the formation of chromosome puffs was observed following heat treatment (Ritossa 1962). Later, the synthesis of distinct proteins, called heat shock proteins (HSP), was described as a consequence of heat shock (Tissieres et al. 1974). Heat shock response and HSP were then also observed in all other organisms including ruminants and it is now considered as one of the main adaptive stress responses of the cell, restoring cellular homeostasis upon exposure to proteotoxic stress, including thermal stress and oxidative stress (Meijering et al. 2015). In spite of being an acute response, researchers found that it improves the survivability of the animal for possible future heat stress episodes (Gaughan et al. 2014), a phenomenon termed as "acquired thermal tolerance" (McClung et al. 2008). The heat shock response confers transient thermal tolerance, in part due to the expression of heat shock proteins (HSP) and related elements, such as the ubiquitin–proteasome system (Velichko et al. 2013). When the response sensitivity and intensity of HSP genes to environmental stressors was observed, HSP70 was found to be the most sensitive to temperature fluctuations and suggested as an important molecular biomarker of heat stress in animals (Dangi et al. 2014a). Heat shock proteins are responsible for maintaining the balance between survival and an effective immune system in the organisms during challenging conditions (Morange 2006). Apart from the expression of heat shock factors (HSFs) and proteins, other components of heat shock response include increased glucose, amino acid oxidation, reduced fatty acid metabolism and activation of endocrine, immune systems via extracellular secretion of HSP (Collier et al. 2008).

Heat shock response is regulated mainly at the level of transcription by four heat shock transcription factors (HSFs), that include HSF1, HSF2, HSF3 and HSF4, which bind to Heat shock elements (HSE) in DNA (Fujimoto and Nakai 2010) resulting in stimulation of HSP expression. The HSF1 to HSF4 has been reported till date in large eukaryotes of which HSF1 has been directly correlated with thermotolerance in livestock (Archana et al. 2017).The HSF1 and HSF3 are activated during heat stress whereas HSF2 is activated during stressors other than thermal stress. The HSF2 is a short lived protein that ensures the continued expression of chaperons acting as inducible regulator when misfolded proteins have been marked for degradation. The coordinated effort of multiple HSFs provides chaperonic coverage to the cellular activities and protects the unfolded proteins. The HSF1 is mainly correlated with induction of HSP70 gene expression (Archana et al. 2017).

Before heat-induced activation, HSF1 exists as a monomer localized to the cytoplasm. Upon heat stimulus, the HSF1 monomer previously bound to HSP during unstressed condition in the cytoplasm, get dissociated and bind with other HSF monomers for trimerization before their nuclear translocation. The heat stress target gene transcription gets activated with the binding of the homotrimeric HSF on HSE in the nucleus hyperphosphorylation resulting in enhanced expression of HSP mRNA (Collier et al. 2008). Thus it requires a certain threshold level of temperature above which the HSP expression will be induced on different livestock species dwelling in different tidal zones which showed different HSP expression (Dong et al. 2008; Tomanek and Somero 2000).

1.1.4 Types of HSP Associated with Heat Tolerance in Ruminant Livestock

Thermotolerance is considered as a quantitative trait which means it is an outcome of many genes involved in this critical biological phenomenon. Based on the molecular weight and biological functions, HSP are classified as HSP110, HSP100, HSP90, HSP70, HSP60, HSP40, HSP10, and small family HSP of which thermotolerance is mainly correlated with HSP70, HSP90, HSP60, HSP105/110 and HSP27 in livestock species (Sharma et al. 2013; Dangi et al. 2014b; Slimen et al. 2016). The HSP70 is one of the most abundant HSP family playing a crucial role in thermal adaptation (Gupta et al. 2013; Banerjee et al. 2014) representing about 2–15% of total cellular proteins expressed by all living organisms and are highly conserved across evolutionary lines (Lindquist 1986; Parsell and Lindquist 1993; Morimoto et al.1994). The HSP expression is considered as a potential indicator of animal adaptation to harsh environmental stressors (Hansen 2004). Though most of the gene expression studies on HSP in ruminants were done using PCR based techniques, some studies include microarrays of blood cells (Salama et al. 2012) and RNA sequencing of milk cells. A study in Mammary Epithelial Cells (MECs) showed that most of the HSP had maximum increase in mRNA

expression at 2–4 h though response was believed to be as early 30 minutes, remained elevated till 12 h post heat stress and eventually declined 16–48 h post heat stress to the level of unstressed MECs with HSP70 being the most predominant form of transcripts induced in buffalo MECs due to heat stress (Kapila et al. 2016).

Amidst the well accepted fact that HSP70 is predominantly involved in thermotolerance, HSP90, HSP110, HSP25 and HSP40 also contribute to the same (Duncan 2005; Kampinga et al. 2003). It was reported that 1–2% of total proteins under non-stress conditions is HSP90 and it is further upregulated under stress by as much as two fold at 37–42 °C (Csermely et al. 1998; Bagatell et al. 2000). A study in broilers and bovine PBMCs showed that heat stress induced expression of HSP90 in the heart, kidney, and liver tissues peaked after a 2-h heat exposure followed by a subsequent basal level expression indicative of its role in acute heat response (Lei et al. 2009; Kishore et al. 2013; Deb et al. 2014). HSP90 is involved in ATP mediated maturation and stabilization of proteins to maintain cellular homeostasis even during severe cellular insults (Jackson 2013). Apart from this, it is also believed to make cells responsive to various stressors by binding to the glucocorticoid receptors, keeping them in their native state until their binding to cortisone, a classical stress hormone (Grad and Picard 2007). It was found that HSP90 forms a multi chaperone complex with HSP70, a protein called Hop and this complex is involved in efficient protein trafficking, removal of damaged proteins via the ubiquitin proteasome pathway (Daniel et al. 2008) apart from the selection of proteins that have undergone oxidative or other toxic damage for ubiquitination and proteasomal degradation (Pratt et al. 2010). This complex could well be the critical factor in deciding which proteins to rescue and which of them to be removed from the cell depending on the ATP reserves, though this hypothesis needs to be investigated.

HSP60 is another protein that is found to be playing a role in cellular thermotolerance. In mammalian cells, 75–80% of HSP60 is in the mitochondria, whereas 15–20% of HSP60 is extra mitochondrial (Soltys and Gupta 1996). A study by Sharma et al. (2013) in goats has shown that HSP60 increased following the animal exposure to 40 °C and its expression was further increased with administration of melatonin. Since melatonin is well known cytoprotectant in heat induced oxidative stress, HSP60 expression with melatonin administration proves the effective role of HSP60 in thermotolerance and it was proved to play a role in refolding of proteins coupled with preventing aggregation of denatured proteins (Dangi et al. 2012). It was also reported in Murrah buffaloes, HF and Sahiwal cows that expression of HSP60 took place at 2 h post heat stress (Kishore et al. 2013). Some researchers have found the expression to be even more quicker at 1 h after exposure to heat stress when goats were subjected to 41–45 °C followed by a decrease in next 2 h, with maintaining constant elevation even at recovery for 6 h post exposure in comparison to pre exposure level.

Whereas, HSP90 and HSP40 are known to work in conjunction with HSP70 to refold proteins, another important protein HSP105 prevents the in vitro aggregation of denatured proteins caused by heat shock just like HSP70 (Yamagishi et al. 2000). In severe heat stress, where ATP levels are markedly depressed, HSP105α and

HSP105β act like substitutes for HSP70 family proteins to suppress the aggregation of denatured proteins (Yamagishi et al. 2003). HSP105α is expressed constitutively and induced by various forms of stress, while HSP105β is an alternatively spliced form of HSP105α that is specifically produced following heat shock at 42 °C (Yasuda et al.1995; Ishihara et al.1999). Dangi et al. 2014a while studying the effect of heat stress on Goat PBMCs proved that HSP105/110 mRNA expression level was almost the same at the initial 5 h; thereafter, it increased with heat exposure and reaching the maximum (about 3.88-fold) at 6 h. Interestingly, HSP60, 70 and 90 were proven to play cytoprotective effect in initial phase of heat stress whereas other HSP like HSP105/110 in the later phases which establishes a characteristic biphasic response to heat stress with respect to HSP expression (Dangi et al. 2014a).

A smaller member of HSP family, HSP27 confers cellular protection under stress conditions and rapidly returns to basal levels once the homeostatic challenge has been removed (Arrigo 2007). Due to changes in phosphorylation and conformation, HSP27 alters its response against heat stress (Theriault et al. 2004). A study in goats has shown increased HSP27 expression in both heat and cold stress conditions making it as one of the candidate genes for assessing thermal stress response of animal (Mohanarao et al. 2014).

Though various HSP were proven beyond doubt to play a cytoprotective role in thermal stress, HSP70 has been the one that caught the larger attention of scientists and was predicted as biomarker of thermal stress tolerance in ruminants. The details of HSP70 are discussed in further sections.

1.1.5 HSP70 as an Ideal Biological Marker to Quantify Heat Stress in Livestock

The most highly conserved family of HSP is the 70 kDa family (HSP70) in eukaryotic ells, which includes 13 genes in human and 4 genes in bovine (Grosz et al. 1992; Gallagher et al. 1993; Kampinga et al. 2009) is distributed in different sub cellular compartments, including cytosol, nucleus, mitochondria and endoplasmic reticulum (Daugaard et al. 2007). They bind to denatured proteins, mediate ATP dependent refolding to their native conformations and prevent aggregation (Lindquist and Craig 1988), thus preventing cellular damage and apoptosis induced by unfolded aggregated proteins (Simon et al. 1995). The inducible form of HSP70 (HSP70i) has been proposed as a predictor or indicator for thermotolerance in the cell (Flanagan et al. 1995). It is well documented that HSP70 is strongly correlated with higher temperature and can be a reliable indicator of thermal stress in cattle (Gaughan et al. 2013).

Members of HSP70 family have many genes like HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA4, HSPA5, HSPA6 & HSPA8 (Daugaard et al. 2007). Heat shock protein 70 is believed to play a crucial role in cytoprotection and can be used as a biomarker of cellular stress with its expression level, an indicator of magnitude and duration of heat stress (Volloch and Rits 1999; Rhoads et al. 2013). There is

considerable evidence that the synthesis of HSP70 is temperature dependent (Zulkifi et al. 2003) and thus HSP70 responses could be considered as cellular thermometer (Nagayach and Prakash 2017). The increased expression of HSP70 following heat stress was reported in many cells like bovine lymphocytes (Patir and Upadhyay 2007; Liu et al. 2010; Mishra et al. 2011), kidney of goat (Zulkifli et al. 2010), myocardium (Gray et al. 2000), lung cells (Fargnoli et al. 1990), hepatocytes (Hall et al. 2000), skin (Maibam et al. 2017) and Peripheral Blood Mononuclear cells of Buffaloes (Manjari et al. 2015) and Goats (Dangi et al. 2014a; Shaji et al. 2016) thereby supporting the view that heat shock proteins provide protection from toxic effects of thermal stress.

Though HSP70 can be considered as cellular thermometer, the precise indication of stress could be puzzling as different researchers found different fold changes of expression working with different cells and some of them are summarized in Table 1.2.

The expression of HSP70i is strictly stress inducible and can only be detected following a significant stress upon the cell or organisms (Satio et al. 2004). Other studies on mammalian species observed that the induction of HSP70 gene expression after cold treatment (4 °C) occurred upon recovery at control temperature (37 °C). The magnitude and the kinetics of the response were, however, related to the duration of cold stress (Liu et al. 1994).

Some researchers found that in bovines the threshold of thermal dose for cellular HSP70 expression was 42 °C (Guerriero and Raynes 1990; Patir and Upadhyay 2010) though a higher level of thermal exposure (>42 °C) of buffalo lymphocytes resulted in reduction of HSP70 expression and decline of cellular viability that was explained by the researchers as failure of the thermoregulatory mechanism to maintain the cellular integrity and homeostasis (Patir and Upadhyay 2010). Apart from elucidating the gene and protein expression in different cells, scientist have also measured the serum/plasma HSP70 levels i.e., extracellular HSP70 or eHSPp70 protein. Though the source of extracellular HSP70 (eHSP70) during heat stress is unclear, the most probable source could be damaged intestinal cells (Shapiro et al. 1986; Doklandy et al. 2006; Lambert 2009; Hom et al. 2012). This extracellular concentrations of HSP tend to be less than the concentration of HSP found in specific organs like liver and muscle (King et al. 2002; Kristensen and Løvendahl 2006; Sørensen 2010) with a little correlation between HSP concentration in muscle tissue and plasma for cattle exposed to heat stress (Kristensen and Løvendahl 2006). Interestingly, a strong correlation is established for eHSP70 with ambient temperature rather than with body temperature indicative of changes in HSP transcription due to intermediate messengers responding to changes in ambient heat instead of body temperature (Horowitz 2001; Gaughan et al. 2014). Few studies exist that measured the levels of eHSP70 in serum during heat stress. In Holstein-Friesian cows the mean (4.46 ± 0.17 ng/mL) and range (0.24 to 26.47 ng/mL) of heat shock protein 72 (HSP72) was reported (Kristensen et al. 2004) whereas in steers of Black Angus breed the estimated mean and range was 5.22 ± 0.62 ng/mL, 0.54 to 19.75 ng/mL respectively (Gaughan et al. 2014). In 77 day old jersey calves the reported values were surprisingly much lower for HSP72 i.e., 0 to 1.3 ng/mL

Table 1.2 Expression pattern of HSP70 for different environmental temperature exposure in different ruminant species/breeds

S.No	Type of study	Species	Breed	Temp-erature	Time	Tissue	Fold Change	Reference
1.	In vivo	Goat	Osman-abadi	40 °C	6 h	PBMC	2.8	Shaji et al. (2016)
						Liver	1.64	Shaji et al. (2017)
						Adrenal	2.03	Shaji et al. (2017)
						Rumen	1.23	Chaidanya et al. (2017)
2.	In vivo	Goat	Sirohi	45 °C – 49.4 °C	4–5 h	Liver	5.94	Rout et al. (2016)
						Spleen	4.96	
						Brain	5.29	
						Kidney	2.63	
3.	In vivo	Cattle	HF	41 ± 1 °C	Summer	PBMC	4.55	Kishore et al. (2016)
		Buffalo	Tarai	41 ± 1 °C	Summer	PBMC	1.73	
4.	In vitro	Cattle	Brahman, Senepol, angus	42 °C	1 h	Lympho-cytes	2–3	Kamwanja et al. (1994)
5.	In vitro	Buffalo	Murrah	37–42 °C	3 h	PBMC	2.5	Mishra et al. (2011)
6.	In vitro	Cattle	Chinese Holstein	42	30 min	BMEC	6	Hu et al. (2016)
	In vitro	Cattle	Chinese Holstein	42	1 h	BMEC	14	
7.	In vivo	Goat	Barbari	41	1 h	PBMC	6.27	Dangi et al. (2014a)
	In vivo	Goat	Barbari	45	1 h	PBMC	6.75	

were reported (Kristensen and Løvendahl 2006) which could be an indication of age related enhancement in thermotolerance whereas Mishra et al. (2011) reported more than 200 times increase in serum HSP70 levels during heat stress in buffalo calves as compared to that of thermo-neutral calves. Though it is expected that eHSP70 is predominantly from damaged intestinal cells, a study on buffalo lymphocytes show that they can produce 1430 ηg HSP70/100 μl (Patir and Upadhyay 2010). Also a study in cultured mononuclear cells of Pelibuey and Suffolk breeds of sheep showed that at 37 and 43 °C, the former breed has a HSP70 concentration of 0.53 ng/ml and 2.85 ng/ml as compared to the latter which had 0.03 ng/ml and 0.53 ng/mL, respectively (Romero et al. 2013).

Apart from the generalized studies on HSP70 as a whole, several studies have also shown the critical roles of its different gene products. Some of the gene products initially were believed to play a role in routine functions in the cell, hence considered as constitutive was later reported to have a role in heat stress response too. The best example being HSPA8, an essential housekeeping gene which was designated previously as Hsc70 as it codes for cognate HSP70 family member plays a role in peptide aggregation, disassembly of large protein complexes and translocation of proteins between cellular compartments (Gething 1997) but was confirmed in later studies that it also plays a role in heat induced cellular response (Mohanarao et al. 2014). Other gene products HSPA1A, HSPA1L, HSPA6 were also found to be elevated in heat challenged cells. Interestingly, HSPA1L was found to decrease in cold stressed cells, a point of further investigation (Mohanarao et al. 2014). HSPA1A and HSPA2 were detected to play a crucial role in guiding conformational status of the proteins during folding and translocation (Arya et al. 2007). In a recent report based on HSPA6 functional studies, it was described that though HSPA6 lacks generic chaperone like properties and was evolved to maintain specific vital functions under conditions of severe stress (Hageman et al. 2011).

It can be inferred from the various studies that once an animal receives significant stress challenge, the HSP response is elicited immediately and gradually wanes with continuous stimulation. This may be due to the fact that HSP70 is only being associated with an initial stress challenge whereby subsequent stress events do not invoke a HSP response to the same extent as the initial stressor. Under moderate heat stress conditions, the HSP response was self-regulated, meaning that HSP expression increased rapidly after initiation of a heat treatment, continued for some time and then decreased to a low rate approximately corresponding to the pre stress rate, a phenomenon termed as attenuation (Lindquist 1980; DiDomenico et al. 1982; Voellmy and Boellmann 2007). Studies in rats (Ogura et al. 2008; Sareh et al. 2011) and humans (Hom et al. 2012; Périard et al. 2012) showed a reduction in HSP70 expression when subjects were exposed to repeated bouts of exercise-induced heat stress. It has been postulated that the reduction in HSP70 expression was due to acclimation to the imposed stressor which was also evident in reduction of eHSP70 over time (Gaughan et al. 2013). An alternative explanation also exists which suggests that HSP70 gene expression in BMEC remained elevated for 4 h at 42 °C and then returned to basal levels after 8 h of exposure, indicating the end of heat tolerance and activation of genes associated with apoptosis (Collier et al. 2008).

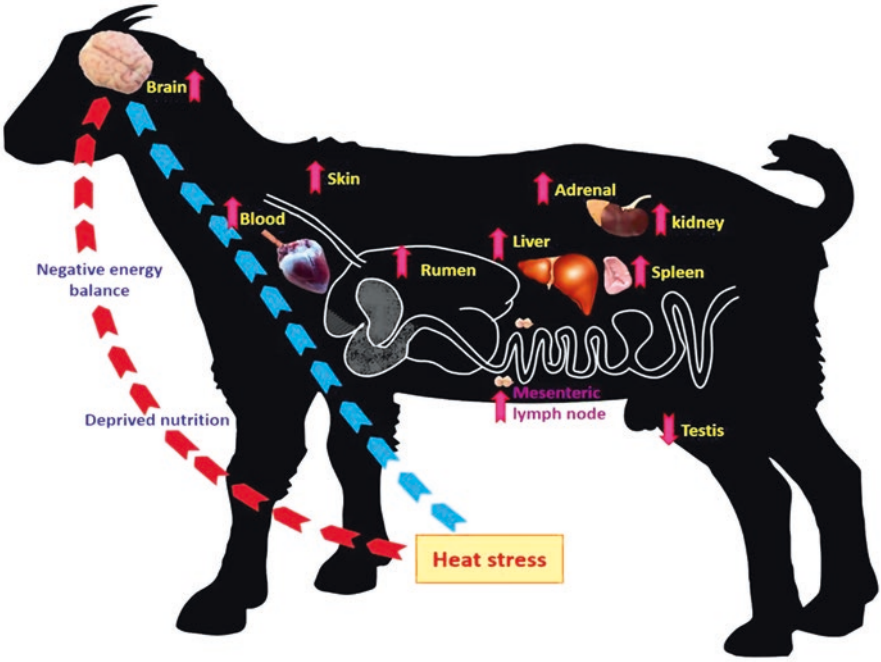

Fig. 1.1 Heat stress impact on HSP70 expression in different tissues of goat

Another notable aspect of thermotolerance is the crosstalk between Nitric Oxide (NO) synthesis and HSP70 expression, since it was found that increased NO synthesis is a stimulus for HSP70 accumulation in adaptation to heat, and the resulting HSP70 has negative effect on activation of NO synthesis thereby hindering its overproduction which may predispose the animal to heat shock induced falling of blood pressure (Malyshev et al. 2000; Hauser et al. 1996). This was indirectly proved by Dangi et al. (2014a) that the HSP expression was not increased during winter season which could be attributed to the fact that the NOS level had not reached to threshold level required to elicit the HSP expression hence establishing a interrelationship between NO and HSP70. Fig. 1.1 describes the expression pattern of HSP70 in different tissues in goats.

1.1.6 Mechanisms of HSP70 Induced Thermotolerance

Thermotolerance is an outcome of many closely interlinked actions both at the cellular level and body system as a whole with the phenomenon being heritable as indicated in studies on bovine embryos of different breeds, where it was proved that lethal effect of thermal stress on blastocyst formation as well as the number of cells

per embryo was comparatively less obvious in the Brahman breed than the Holstein or Angus breeds of cattle (Paula-Lopes et al. 2003).

These tolerance mechanisms though depend on, but are not entirely mediated by HSP. Though HSP70 is considered as a marker of heat and humidity stress (Manjari et al. 2015), there are several studies suggesting that thermotolerance can be induced with a delayed HSP response (Shabtay and Arad 2005), without the induction of HSP synthesis (Widelitz et al. 1986; Boon-Niermeijer et al. 1987) or that induction of HSP may be uncoupled from acquired thermotolerance (Easton et al. 1987; Smith and Yaffe 1991). Since thermotolerance implies the maintenance of near normal physiological functions of the cell which depends upon the homeostatic metabolism involving many proteins, HSP70 is primarily found to regulate protein homeostasis and promote cell survival (Hartl and Martin 1995). There are studies which asserted that over expression of HSP provide protection against hyperthermia, circulatory shock and cerebral ischemia during heat stroke which signifies the central role of HSP in cytoprotection (Ganaie et al. 2013) that can be supported by the fact that 15% or more of total cellular proteins during heat stress are HSP as compared to basal value of 5% (Pawar et al. 2014). It binds to nascent peptide chains on ribosomes, protecting the hydrophobic surface that would normally be exposed to solvent, thus preventing aberrant folding or aggregation, until the whole peptide chain is synthesized and proper folding occurs (Alberts et al. 2002). All HSP70 and related proteins bind ATP with high affinity that results in acceleration of binding and release of polypeptides (Chappell et al. 1986; Fung et al. 1996). Formation of dimers and higher-order oligomers of HSP70 has been suggested, with the monomeric protein representing the functionally active chaperone (Nemoto et al. 2006; Angelidis et al. 1999).

There are various other mechanisms by which HSP70 confers cellular protection during heat stress. Though many of these mechanisms were taken from human studies, these can well be extrapolated to general mammalian cells with prospects for future research in ruminant specific studies to look for fine regulations. HSP70 binds with high affinity to the plasma membrane, eliciting a rapid intracellular Ca^{2+} flux, activating NF-kappa, and upregulating the expression of cytokines in human monocytes (Asea et al. 2002). A study on buffaloes showed that with the increase of HSP70 there was a proportionate increase in the level of TNF-α, IL-12 and GM-CSF, indicating the representative role of HSP70 as a cytoprotectant under stress (Pawar et al. 2014). Studies revealed that HSP play a regulatory role in various types of immunity and augmentation of HSP-derived peptides in buffalo lymphocytes increased the innate and adaptive immune effectors (Mishra et al. 2011). HSP70 also regulate the adaptive immunity as it presents the peptide fragments from stressed cells to cytotoxic T lymphocytes (Verdegaal et al. 1996; Basu 2001). Apart from the animal health aspect, HSP70 also seems to be contributing for better meat quality, since a study in goats found that HSP70 provides protection to muscle glycogen content, the one responsible for favorable pH post slaughter thereby influencing meat quality (Zulkifli et al. 2010). The protective effect of HSP70 in different organs such as the heart and kidney tissue has been well established (Latchman 2001). There are strong opinions regarding the lesser efficacy of HSP70

expression in the in vitro systems as compared to in vivo. In vitro heat stressed lymphocytes have minimal increase in HSP70 level compared with in vivo exposure which demonstrates multiple level of complexity in HSP70 expression and function in vivo (Mishra et al. 2010).

1.1.7 Species and Breed Variations in HSP70 Gene and Its Expression Among Ruminants

The basic doctrine that species vary in terms of adaptation to their natural environment has created intense interest among scientific community to find out the causes behind such variations. Differences exist between species and breed with regard to the ability to reduce metabolic and endogenous heat production and increased heat dissipation in domesticated ruminants (Silanikove 2000a; Collier et al. 2005). Among species, sheep and goats are considered less susceptible to heat stress than cattle (Silanikove 2000a, b). Scientists are of strong opinion that degree to which an animal resists change in body temperature varies with different breeds and species because of differences in their heat regulating mechanisms (Salah et al. 1995). It has also been postulated that differences in HSP concentrations between individuals (Gaughan et al. 2014), species (Agnew and Colditz 2008) and breeds (Dangi et al. 2012; Singh et al. 2014; Manjari et al. 2015; Maibam et al. 2017) may be due to differences in thermotolerance. Goats have a wide range of ecological adaptability and are more productive in harsh environment than other ruminants including sheep (Devendra 1990; Shkolnik and Silanikove 1981). They have well developed adaptive mechanisms that allow their survival at very high (45–50 °C) as well as very low temperatures (−20 to −40 °C) (Nagayach et al. 2017). Among the HSP, HSP70 has a significant role in cellular thermotolerance (Barbe et al. 1998) and animal survival (Barbe et al. 1998; King et al. 2002). Goats are considered more tolerant to high THI values compared to dairy cows because of their metabolic size and high water conservation capacity (Silanikove 2000b). Similarly, Holstein embryos are more affected by heat stress than Brahman preimplantation embryos, which proves that inheritance of resistance is shown in dairy cattle (Shimizu et al. 1996).

There is evidence that bovines that evolved in tropical climates have acquired genes to protect cells from the deleterious actions of high temperatures (Hansen 2004). For example there are differences between Brahman and Holstein in endometrial responses to culture at elevated temperature (Malayer and Hansen 1990). The availability of genome sequences of various species has helped in analysis of variations in critical genes responsible for thermotolerance. For example it was analyzed that entire nucleotide sequence of goat HSP70–1 gene shows 97.8% homology with cattle, 96.3% with buffalo, 99.4% with sheep (partial), 97.5% with yak, 95.3% with pig, 94.4% with horse, and 94.1% with human indicating close evolutionary relationship. Similarly, inferred amino acid sequence of 641 residues of goat HSP70 gene was 100% similar to sheep (partial), 98.6% to cattle, 95.9% to buffalo, 98.4% to yak, 98% to pig, 98.1% to horse, and 97.7% to human sequence

(Gade et al. 2010). Some of the regions like amino acid sequences 9–16 and 131–139 were found to be highly conserved in the HSP70 family of proteins (Gutierrez and Guerriero 1995). In goat HSP70, amino acid isoleucine is replaced by threonine at position 9, and sequences from 10–16 and 131–139 were found to be conserved. The amino acid substitution pattern of different HSP indicated that HSPA6 and HSP1L have evolved independently in mammalians compared to other HSP (Banerjee et al. 2014).

Apart from the sequence similarities between different species, some of the researchers also proposed the possibilities of Single Nucleotide Polymorphisms (SNPs) with the aim of exploiting it for selection of thermotolerant animals. Studies also revealed the association between SNPs in HSP with respiration rate and body temperature (Deb et al. 2013). A study on SNPs in HSP70 of Zebu and Temperate cattle revealed SNPs at 3′ untranslated region (UTR) of HSP70 gene (Adamowicz et al. 2005) and five novel mutations were also found in HSP70 gene arising the probability of thermotolerant genotypes (Li et al. 2011). Lamb et al. (2007) found 8 SNPs in HSP70 gene of different cattle breeds and deduced that 5 of them were related to Brahman ancestry. In one of the studies, it was found that the allelic variants of HSP70 gene were found to be associated with heat tolerability of the Tharparkar cattle (Bhat et al. 2016). The relationship between inducible HSP70.1 SNPs and the Heat shock response of PBMCs was studied in Italian Holstein dairy cows which showed the presence of SNPs in the 5′-UTR region of inducible HSP70 that were well correlated with thermotolerance. This also led to the opinion that mutation sites would be serving as the appropriate molecular genetic markers for thermal tolerance and the differential rate of HSP mRNA expression was observed between *Bos indicus* and *Bos taurus* embryos produced in vitro subjected to Heat stress (Silva et al. 2012). Similarly, the coding sequences HSPA1A gene were analyzed in two breeds of cattle Deoni (Zebu type) and Holstein Friesian that proved 7 SNPs, including 5 transitions and 2 transversions in the Deoni breed of cattle, while 5 SNPs, including 2 indels, 2 transitions, and 1 transversion, were observed in HF crossbred cattle, indicating a high degree of genetic variability in the HSPA1Agene in the breeds (Kerekoppa et al. 2015).

Though there is wide spread acceptances regarding the polymorphisms in gene difference of opinion exist regarding the promoter variation and its role in differences in the expression of HSP70. Some workers analyzed 5′ flanking region of HSP70 for cis-acting sites in both zebu and exotic cattle, and concluded that promoter variation may not be the source of the difference in expression level of HSP70 (Behl et al. 2014) whereas other reports suggests that the polymorphism in the promoter region as a reason for the variation in HSP70 mRNA, HSF1 mRNA expression level, and apoptosis and hence these mutation sites can yield as useful genetic molecular markers against HS in cow (Cai et al. 2005). Also, polymorphism at the 5′UTR of HSP70 was associated with the increased viability of PBMC as well as higher expression level of protein (Basiricò et al. 2011). Similar relationships were observed too between cellular thermo tolerance and milk production traits (Deb et al. 2013). In an effort to understand the effects of thermal stress on bovine mammary development, Collier et al. (2006) observed an augmented expression of

a large number of genes in cultured bovine mammary epithelial cells (BMEC). Kamwanja et al. (1994) reported that *Bos indicus* (Brahman) cattle showed higher survivability of lymphocytes than that of *Bos taurus* under heat stress, however the HSP70 level of expression showed no difference between the two breeds. Interestingly, the increased HSP70 expression was found to be associated with shorter productive life of cattle and reproductive parameters including pregnancy rate, weaning weight of calf and fertility in dairy cattle (Schwerin et al. 2003; Starkey et al. 2007; Rosenkrans et al. 2010) which in whole indicated the significance of SNPs at promoter elements of HSP70 that can be used as one of the reference to be added for selecting dairy cattle in terms of thermo adaptability (Deb et al. 2013).

In vitro heat stress is a well established mode of detecting HSP gene expression in cultured PBMC. In one of the studies, it was proved that PBMCs from Sahiwal cows were better able to survive incubation at temperatures simulating hyperthermia at 42 °C or hypothermia at 4 °C than Frieswal cows (Bhanuprakash et al. 2016). Paula-Lopes et al. (2003) reported that the apoptosis percentage of PBMC cells after heat challenge was significantly lesser in samples obtained from Brahman and Senepol cows than Angus and Holstein counterparts. Similarly, the mRNA transcript was higher in Sahiwal than Frieswal immediately after post heat or cold stress to 6 h post recovery time at 37 °C (Bhanuprakash et al. 2016).

1.1.8 Seasonal Variations in HSP70 Expression

The protective role of HSP in thermal stress of domestic ruminants is indicated in many of the studies which mean there exist seasonal variations in the HSP expression. A study in skin cells revealed that relative expression of both the constitutive HSP (HSP70.8) and inducible HSP (HSP70.1, HSP70.2) genes increased during summer and winter season compared to spring in Tharparkar and Karan Fries breeds of cattle with highest being in summer. Interestingly, in summer season, the magnitude of expression of constitutive HSP (HSP70.8) was higher in Tharparkar than Karan Fries, and that of inducible HSP (HSP70.1 and HSP70.2) it was vice versa (Maibam et al. 2017). These findings may consolidate the belief that constitutively expressed HSP in skin cells act as a natural barrier against potential environmental stressful attacks eliciting heat acclimation and reducing the threshold for systemic heat shock response (Trautinger et al. 1993; Horowitz 2001). Similar findings were also reported by Singh et al. (2014) in skin fibroblasts of Karan Fries, a crossbred and Tharparkar, a zebu cattle when exposed to heat stress and attributed this differences to their difference in the adaptive responses of skin to heat stress between the breeds. The protective response of the skin against deleterious effect of heat stress was found to be higher in Tharparkar than Karan Fries, since Tharparkar showed greater expression of constitutive HSP (HSP70.8) than Karan Fries during summer season.

Further, greater expression of inducible HSP during summer in Karan Fries than Tharparkar indicated that skin of crossbred cattle was under more stress than that of

zebu cattle during summer heat stress as it was opined that higher expression of inducible HSP is the indication of more deterioration due to heat stress (Singh et al. 2014; Maibam et al. 2017). Hansen (2004) reported a direct correlation between quantum of inducible HSP expression and protein denaturation in heat stressed cattle. Many studies in various types of cells like lymphocyte (Kamwanja et al. 1994), PBMCs (Lacetera et al. 2006) and dermal fibroblasts (Singh et al. 2014) found that thermotolerant breeds expressed less HSPA1A when subjected to heat shock. Alternatively, breeds exhibiting higher mRNA levels of HSP70 are better at regulating heat stress though the species referred in this condition is goat and the comparison was among tropical goats. Research done on goat adaptability to heat stress found that Sirohi breed had a higher mRNA level of HSP70 gene indicating that it was better at regulating heat stress compared Barbari, Jamunapari and Jakhrana breeds (Rout et al. 2016) though the earlier study by Banerjee et al. (2014) showed no difference between Sirohi and Barbari. Both schools of thought seem logically correct with former being an indicative of higher threshold for eliciting a response and the latter being higher magnitude of response. The reports of breed variation and its interpretation of thermotolerance though depends on lot of factors like control gene for normalization (Derveaux et al. 2010), the type of cells used and probably also the age of sampled cells. With respect to type of cells, HSP70 mRNA expression was found to be higher in the liver, kidney as compared to other tissues (Nagayach et al. 2017).

Relative expression of HSP70 genes varied markedly among the heat- and cold-adapted goat breeds with a moderate variation between breeds and showed a good response to increased or decreased ambient temperature. The expression of HSPA8, HSPA6, and HSPA1A was higher, but the relative mRNA expression of HSPA1L and HSPA2 was very low. Results indicated that the expression level of HSPA8 and HSPA1A was higher during both winter and summer. The expression level of HSPA6 and HSPA1L was higher only during summer. HSPA2 was observed to be down regulated during the summer and winter seasons. During summer, the relative expressions of all the HSP70 genes were higher in cold-adapted breeds like Gaddi and Chegu than the heat-adapted breeds like Sirohi and Barbari. The HSPA8 and HSPA1A expression during winter in heat-adapted breeds was observed to be higher than cold-adapted breeds (Banerjee et al. 2014). The quantitative analysis shows that the fold increase expression in heat challenged PBMCs with respect to HSPA1A, HSPA1L, HSPA6, HSPA8 and HSP27 were 19, 3.3, 16.6, 14.4 and 6.4 times, respectively, as compared to control cells whereas the mRNA expression levels of HSPA1A, HSPA2, HSPA6 and HSPA8 genes showed no variation between cold challenged and normal PBMCs with reduction in HSPA1L was observed in cold challenged cells (Mohanarao et al. 2014) .

Increase of HSP70 gene expression during summer was observed to be higher in cold-adapted goat breeds (Gaddi and Chegu) as they experience higher stress during summer than heat-adapted goats (Sirohi and Barbari). There is difference of opinion regarding the increase in expression of HSP70 family during cold stress. Some believe there is no significant increase (Singh et al. 2014; Jagan et al. 2014) where as others believe in contrary (Banerjee et al. 2014; Kumar et al. 2015). In a study

during winter it was reported that the expression of HSP70 genes was higher in heat adapted goats than the cold-adapted goats (Banerjee et al. 2014) which was not surprising since cold-induced denaturation of proteins is also reported (Kostal and Tollarova –Borovanská 2009) and in turn, partially denatured or misfolded proteins are potent triggers of rapid HSP70 accumulation (Feder and Hofmann 1999).

An age-related decrease of HSP expression was reported in Cattle (Gaughan et al. 2014). In contrast, Kristensen et al. (2004) reported that younger cows (<305 d of age) had reduced HSP72 concentration compared with older cows (305 to 560 d of age). The correlation of HSP70 expression with age is problematic since the reduction might also be attributed to heat acclimation with time resulting in lesser response with age (Gaughan et al. 2014). Another possible reason could be the age, health and season related antioxidant status of the body since it was found that, antioxidants administration decreased the HSP expression in PBMCs. It is well proven that vitamin E is the first line of defense against lipid peroxidation and when the concentration of antioxidant vitamins decreases, lipid peroxidation increases in the plasma and tissues, leading to damage in the integrity of cell membranes (Mcdowell 1989). There is a strong relationship between lipid oxidation and HSP70 synthesis in stressed cells (Mahmoud et al. 2004).

1.2 Conclusions and Future Perspectives

Thermal stress, especially heat stress is a major problem for the ruminant farming across the globe. Though there are strategies to mitigate the effects of heat stress in ruminants, the most apt long term strategy should be to enhance the thermotolerance ability in animals. Extensive studies revealed that HSP play a crucial role in heat stress tolerance with the lead role given to HSP70. This chapter focuses various aspects of HSP70 especially related to its mechanisms, variations with respect to species, breed, season and age etc. The differences in proven molecular variations like SNPs in HSP70 gene and promoter regions will serve as future tools for marker assisted selection of thermotolerant breeds specific for a climate. This chapter also focuses on how inter-molecular relations exist between HSP70 and other HSP with respect to cytoprotective effects. More importantly, the chapter also throws light on biphasic response of heat stress in animals. Though there are still a lot of un answered questions exist with respect to the precise role of HSP70 in heat stress, as of now it can be assumed that it is one of the efficient biological marker of selecting animals for developing the breed stock that can act as possible parents for future offsprings.

There is a wide spread agreement across the world that climate resilient animal agriculture is the need of the hour. It demands the necessity of animals that can with stand thermal stress especially heat stress but still can have optimum production. The selections of animals require suitable biomarkers and HSP70 fits into this category efficiently. The future studies should concentrate on how the interactions of HSP70 takes place with other HSP and which of the gene products are respon-

sible for critical cytoprotective functions. To have wider applications, the information must be generated species and breed wise. The studies are also required on finer aspects of the characterization with respect to qualitative and quantitative differences in expression of various HSP70s apart from unleashing the roles of its different domains. Also the cross breeding programs in future should take into consideration not only the productive parameters but also the thermotolerance ability so that resultant crossbreds have beneficial phenotypes. In order to strike a crucial balance between production and thermotolerance in ruminants, HSP70 will be of utmost importance and future interactive studies between HSP70 and other genes are going to be vital for its use as selection tool for the future breeding programs.

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Chapter 2
Expression Dynamics of Heat Shock Proteins (HSP) in Livestock under Thermal Stress

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Abstract Increased ambient temperature increases heat gain by animal which results in heat stress and reduced performance, leading to decreased efficiency of livestock farming. In addition to adaptive biochemical, endocrine and physiological responses, the molecular events that underlie thermotolerance involve the coordinated synthesis of series of heat stress responsive genes, which are responsible for amelioration of deleterious effects of heat stress. Heat Shock Proteins (HSP) are the key players in the adaptive responses to the stress. Intracellular HSP70 confers cytoprotection against thermal and oxidative stress induced cellular damage. Heat Shock Factor (HSF) are the regulatory proteins that is activated by heat stress and control transcription of HSP by binding to Heat Shock Elements (HSE) in HSP genes. Heat shock causes profound modulation in cell signaling pathways that lead to transcription of Nitric oxide synthases (NOS), Toll like receptors and Interleukins. Studies on heat stress in livestock and model animals indicate that TLR 2/4 and IL 2/6 possibly play a vital role via activation of the JAK-STAT pathway. Crosstalk between HSP90, iNOS and eNOS play an important role in mitigating thermal insults and confer thermo tolerance during long term heat stress exposure in livestock. Recent study indicates important roles of Vitamin C, Vitamin E plus Selenium and Betaine as an antioxidant in maintenance of cellular homeostasis. Positive correlation has been found between melatonin and HSP, which explains its importance in heat stress adaptation. These mechanisms possibly work in an orchestrated manner to

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minimize the devastating effect of heat stress and play pivotal role in the thermotolerance by blocking heat stress-induced cellular death, which helps livestock in acclimation to heat stress.

Keywords Betaine · Cytoprotection · Heat Shock Proteins · Heat Stress · Immune Response · Interleukins · Nitric oxide synthases · Oxidative Stress · Selenium · Toll like receptors · Vitamin C · Vitamin E

Abbreviations

20S	20S proteasome
Apaf-1	Apoptotic protease activating factor - 1
APC	Antigen presenting cells
ATP	Adenosine triphosphate
CD14	Cluster of differentiation 14
CTL	Cytotoxic lymphocyte
DAMPs	Damage associated molecular patterns
DC	Dendritic cells
DNA	Deoxyribonucleic Acid
E2	Ubiquitin conjugating enzyme
eHSP	Extracellular heat shock proteins
eNOS	Endothelial nitric oxide synthases
FBS	Fetal bovine serum
Gp96	Glucose regulated protein 96
GPx	Glutathione peroxidase
GR	Glucocorticoid receptor
GSH	Glutathione
HL-60	Human promyelocytic leukemia 60
HR	Heart rate
HS	Heat stress
HSE	Heat shock elements;
HSF	Heat shock factor;
HSP	Heat shock proteins
htpG	High temperature protein G
iHSP	Intracellular heat shock proteins
IL	Interleukins
iNOS	Inducible nitric oxide synthases
JAK-STAT	Janus Kinase – signal transductor and activator of transcription
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
MAPK	Mitogen activated protein kinase

mM	Millimolar
mRNA	Messenger ribonucleic acid
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	Non-heat stressed
NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthases
OH	Hydroxyl
PAMPs	Pathogen associated molecular patterns
pAPCs	Professional antigen presenting cells
PBMC	Peripheral blood mononuclear cells
PRRs	Pattern recognition receptors
qPCR	Quantitative polymerase chain reaction
RIP-1	Receptor interacting serine/threonine protein 1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Respiratory rate
RT	Rectal temperature
Se	Selenium
SOD	Superoxide dismutase
THI	Temperature humidity index
TLR	Toll like receptors
TMI	Transition metal ions
TNF-α	Tumor necrosis factor-α
UBI	Ubiquitin
μM	Micromolar
pAPCs	Professional antigen

2.1 Introduction

Stress is the result of environmental forces continuously acting upon animals which disrupt homeostasis resulting in new adaptations that can be detrimental or advantageous to the animal (Stott 1981) depend on the prevailing situation. Many stressors like environmental, climatic, nutritional, physical, social or physiological affect welfare and performance of animals. Increased ambient temperature may lead to enhanced heat gain as compared to heat loss from the body and may cause heat stress in animals. Environmental heat or heat stress is the most detrimental to dairy animals and results in the hindrance of feed consumption, decreased milk production and reproductive performance. Thermal stress triggers a complex program of gene expression and biochemical adaptive responses (Lindquist 1993). Acclimatization to heat stress requires the physiological integration of many organs systems viz. endocrine, cardio-respiratory and immune system (Altan et al. 2003). Acclimation to

heat is a biphasic process: transient perturbed phase and a long lasting period in which acclimatory homeostasis is developed (Horowitz et al. 2004). It alters the expression of preexisting features that are beneficial to the organism in a particular environment and as a life time process, persisting stress improves the fitness of an individual population to tolerate that stress. Thermo tolerance in animals during prolonged heat stress is characterized by the heat shock response and adaptations associated with acclimatization. Biologically, the ability to survive and adapt to thermal stress appears to be a fundamental requirement of cellular life, as cell stress responses are ubiquitous among both eukaryotes and prokaryotes and key heat shock proteins (HSP) involved in these responses are highly conserved across evolutionary lines (Parsell and Lindquist 1993) and synthesized during heat stress (Lindquist and Craig 1988) to protect cells from toxic effects of heat and other stresses (Pockley 2001). However, cellular response to heat shock involves not only HSP but also several other bio-molecules (Kregel 2002). HSP and NOS are two families of proteins that seem to be particularly involved in the adaptive responses to the stress (Renis et al. 2003).

2.1.1 Impact of Heat Stress on Cellular Functions

The heat stress (HS) response is a highly conserved cascade of protein activation and altered gene expression in response to a variety of stressors (Collier et al. 2008). Cellular exposure to elevated temperature induces a number of anomalies in cellular functions which include a general inhibition of protein synthesis, defects in protein structure and function, morphological changes due to cytoskeleton rearrangements, shifts in metabolism, alterations in cell membrane dynamics and fluidity, and decrease in cell proliferation. These anomalies invoke large changes in gene transcription and protein synthesis known as the HS response (Lanks 1986). The timing and success of these alterations ultimately determines cell survival and acclimation or cell death. Hyperthermia at the cellular level exhibit substantial variation due to type of cell and tissue. For example, one cell type develops thermal tolerance at a given thermal load, whereas; another cell type readily undergoes apoptosis at the same temperature. Despite these inequities, high ambient heat load will elicit reproducible changes at the transcription factor level. Heat shock factor (HSF) which is part of transcription factor family has been known the first responders during the onset of elevated cell temperature (Trinklein et al. 2004; Page et al. 2006). These transcription factors coordinate the cellular response to thermal stress and affect expression of a wide variety of genes including HSP (Akerfelt et al. 2007).

Regulation of HSP gene transcription is mediated by the interaction of the HSF transcription factors (of which the principal one in vertebrates is HSF-1) with heat shock elements (HSE) in the HSP gene promoter regions (Voellmy 1994; Morimoto et al. 1994). The HSE is a stretch of DNA located in the promoter region of susceptible genes containing multiple sequential copies (adjacent and inverse) of the con-

sensus penta-nucleotide sequence 5′-nGAAn-3′ (Morimoto and Santoro 1998) and has been found in both HSP and in a number of other genes. HSF-1, HSF-2, and HSF-4 are present in mammalian systems (Morimoto and Santoro 1998) and HSF-3 is present in avian species but not in humans. HSF-1 is involved in the acute response to heat shock; the others are involved in a number of different regulatory and developmental processes and, are not generally thought to play roles in the cellular response to heat (Morimoto and Santoro 1998; Pirkkala et al. 2001). Heat shock can produce reversible inactivation of HSF-2 (Mathew et al. 2001). HSP are also present in cells under perfectly normal conditions to maintain assigned regular functions. In the unstressed state, HSF-1 is present in the cytoplasm as a latent monomeric molecule that is unable to bind DNA. The exposure of hydrophobic domains of denatured proteins during heat stress appears to be the initial stimulus for activation of HSF-1 which separates them from HSP and HSP preferentially bind to denatured proteins. Under stressful conditions, HSF-1 is hyper-phosphorylated by a member of mitogen activated protein kinase (MAPK) (Knauf et al. 1996; Kim et al. 1997). HSF-1 is phosphorylated under stressed condition with a capacity to bind DNA, and translocates from the cytoplasm to the nucleus (Morimoto and Santoro 1998 and Pockley 2003). The central process of HSF-1 activation is the equilibrium between the bindings of free HSP molecules as HSP70 to the HSF-1 and to stress mediated unfolding proteins (Snoeckx et al. 2001). After activation by thermal stress, HSF-1 is found primarily in the nucleus in trimeric form, concentrated (in human cell lines) in granules (Sarge et al. 1993). It is this activated trimeric form of HSF-1 that binds to the HSE and is involved in increased HSP gene transcription during heat stress (Sarge et al. 1993). HSP are synthesized in the cytoplasm on ribosome assembly and bind to denaturing proteins to renature them. Although HSF-1 has traditionally only been associated with regulation of HSP, recent evidence now links it to regulation of carbohydrate metabolism, transport, cytoskeleton, and ubiquitination during HS (Page et al. 2006). The HSF-1 gene has been mapped to chromosome 14 in cattle (Winter et al. 2007); however, investigations of HSF-1 regulation and function are limited in bovine species despite the importance of HSF-1 to the initiation of the HS response.

2.1.2 Impact of Endocrine System on Cellular Heat Shock Response

The importance of the endocrine system mediating acclimation to environmental stress, growth, and metabolism is clear and therefore it is not surprising that thermal stress has a profound effect on circulating hormones. Many thermal stress-induced hormonal alterations have been known for several decades. For example, hyperthermia depresses thyroid function, presumably to depress metabolic heat generation.

Table 2.1 Hormones affecting Heat Shock Protein (HSP) gene expression or protein activity

Hormone	Effect	Reference
Melatonin	Increased HSP gene expression	Sharma et al. (2013)
	Increased HSP gene expression in pancreatic AR42J cells	Bonior et al. (2005)
Leptin	Down regulated HSP 70 in chicken liver and hypothalamus	Figueiredo et al. (2007)
IGF-I	Increased HSP in epidermis of IGF transgenic mice	Shen et al. (2007)
Prostaglandin A	Increased expression of HSP in bovine mammary epithelial cells, human K562 cells, and human monocytes	Collier et al. (2007); Amici et al. (1992); Ella et al. (1999)
Insulin	Stimulated HSP gene in cardiac tissue	Li et al. (2006)
Growth hormone	Stimulated HSP in whole blood of sea bream	Deane and Woo (2005)
Estrogen and androgens	Increased HSP gene expression in human neurons	Zhang et al. (2004)
Glucocorticoids	Increased cytosol HSP and HSP gene expression	Vijayan et al. (2003)
Prolactin	Stimulated HSP 60 in rodent luteal cells	Stocco et al. (2001)
Vasopressin	Stimulated HSP in renal tubular cells	Xu et al. (1996)
Catecholamines	Stimulated HSP in brown adipose tissue	Matz et al. (1996)

Two hormones known to increase in plasma in response to thermal stress (prolactin and glucocorticoids) are associated with modifying the intracellular HS response. When glucocorticoids enter the cytoplasm they cause the release of preformed cytoplasmic HSP70 and HSP90, which are bound to the glucocorticoid receptor. This preformed HSP constitutes the first line of defense against HS because the proteins are already in the cytoplasm and do not require synthesis. Release of the HSP in response to increased glucocorticoid binding to its receptor provides an instant pool of HSP to protect against protein denaturation. The hormone-receptor complex then moves into the nucleus where, among other actions, it enhances expression of HSP genes (Vijayan et al. 2003). Prolactin also modifies HSP gene expression. Stress-response hormones (glucocorticoids) are elevated during initial HS exposure and then become depressed with prolonged periods of thermal stress.

The cortisol concentration were significantly up-regulated in pig (Ju et al. 2014), cattle (Abilay et al. 1975; Collier et al. 2007; Bharati et al. 2017c) during acute heat stress while prolonged heat exposure has been shown to down regulate the cortisol concentration in cattle (Christison and Johnson 1972; Collier et al. 2007) and yak (Sarkar et al. 2007). The increase in cortisol concentration is an indicator of stress, which during acute heat exposure period might promote gluconeogenesis process to avail glucose while the decline in cortisol concentration during chronic heat exposure period might help to reduce the metabolic heat production in animals.

As stated earlier, the process of acclimation is under endocrine regulation. It is now clear that a variety of endocrine or paracrine signals associated with a range of stressors modify the intracellular HS response (Table 2.1).

2.1.3 Thermal Stress and Expression of HSP

A hallmark of the "stress response" is the up regulation of HSP. HSP were first discovered in 1962 (Ritossa 1962) as a set of highly conserved proteins whose expression was induced by different kinds of cellular stresses, such as hyperthermia, oxidative damage, physical injury or chemical stressors. In mammalian cells, non-lethal heat shock produces changes in gene expression and in the activity of expressed proteins, resulting in what is referred to as a cell stress response (Jaattela 1999; Lindquist 1993). This response characteristically includes an increase in thermo-tolerance (i.e. the ability to survive subsequent, more severe heat stresses) that is temporally associated with increased expression of HSP. Heat-induced changes in gene expression occur both during hyperthermia as well as after return to normothermia. HSP are intracellular molecules, involved in stabilizing cells during thermal exposure (Hendry and Kola 1991). These HSP have a protective function and a strong correlation between their induction and an increase in thermo tolerance has been observed (Lindquist and Craig 1988).

HSP perform versatile roles in cells, from housekeeping duties under unstressed conditions, including regulation of protein quality control, to life and death decisions following cellular stress, via their interaction with members of the apoptotic cell death cascade (Kalmar and Greensmith 2009). Mammalian HSP have been classified into major families according to their molecular size: HSP60, HSP70, HSP90, HSP105/110 and the other small HSP. Each family of HSP is composed of members expressed either constitutively or regulated inducible, and/or targeted to different sub-cellular compartments. For example HSP60, HSP70 or HSP90 are constitutively expressed in mammalian cells while others, HSP27 and HSP70, are strongly induced by different stresses, such as heat, oxidative stress, or anticancer drugs. HSP70 family is present in every cell type and tissue under both unstressed and stressed conditions. HSP70 is induced by physiological stressors, pathological stressors, and environmental stressors (Kiang and Tsokos 1998). Some of the important housekeeping functions attributed to the molecular chaperones include import of proteins into cellular compartments, folding of proteins in the cytosol, endoplasmic reticulum and mitochondria, degradation of unstable proteins, dissolution of protein complexes, prevention of protein aggregation, protein translocation across organelle membranes, control of regulatory proteins and refolding of mis-folded proteins (Bakau and Horwich 1998). The inducible HSP expression is regulated by the HSF. In response to various inducers such as elevated temperatures, oxidants, heavy metals, and bacterial and viral infections, most HSF acquire DNA binding activity to the HSE, thereby mediating transcription of the heat shock genes, which results in accumulation of HSP (Wu 1995; Morimoto and Santoro 1998; Morano and Thiele 1999). Members of HSP60, HSP70, and HSP90 families have all been linked with innate immune stimulation (Osterloh and Breloer 2008; Srivastava 2002; Murshid et al. 2011). Various cell lines have been investigated for their ability to bind HSP, including mostly professional antigen presenting cells (pAPCs) such

as dendritic cells (Reed and Nicchitta 2003), macrophages, and peripheral blood monocytes (Sondermann et al. 2000).

The protective role of HSP is usually confined to their chaperone function; that is, their capacity to bind denatured proteins and thus prevent their irreversible aggregation (Lindquist 1993). A molecular chaperone is any protein that interacts with and aids in the folding or assembly of another protein without being part of its final structure. The majority of chaperones prevent the aggregation of "sticky" protein folding intermediates. By binding to their targets, chaperones are acting as "collectors" of damaged proteins. Chaperones are classified into different groups on the basis of sequence homology. Many are stress proteins as their synthesis is induced under conditions of stress (e.g., heat shock or oxidative stress), which structurally destabilize a subset of cellular proteins. Besides their fundamental role in *de novo* protein folding, chaperones are involved in various aspects of proteome maintenance, including assistance in macromolecular complex assembly, protein transport and degradation, and aggregate dissociation and refolding of stress-denatured proteins.

Upon stress the most prominent HSP present in the nucleolus are the inducible HSP70 and HSP110 (Welch and Suhan 1986). The fact that some HSP translocate to the nucleolus suggests a specific and unique role in the repair and protection of these cellular structures (Collier and Schlesinger 1986). Upon recovery after heat shock, HSP70 exit the nucleolus to accumulate back in the cytoplasm, more specifically in the perinuclear region, along the perimeter of the cell, and in association with large cytosolic phase dense structures (Welch and Framisco 1984; Welch and Suhan 1986). Perinuclear condensation of HSP70 seems to coincide with reassembly of the centrosome and microtubuli, and also with the cytoplasmic distribution of ribosomes. This suggests that HSP70 plays a crucial role in the function of these organelles immediately after heat shock and during subsequent recovery phase (Brown et al. 1996). Proteins that lose their normal three-dimensional conformation provoke HSP synthesis through the activation of HSF. During and after heat shock, cytosolic proteins normally aggregate and have a reduced solubility (Vidair et al. 1996). Along with its release during heat stress they themselves are subject to strict autoregulation by multiple molecular mechanisms (Lindquist 1993). HSP chaperoning is a permanent cellular event during both nonstressed and stressed conditions. However, during heat shock or other stresses, upregulation of the synthesis and translocation of various HSP to other cellular compartments suggest that during evolution, tissues develop intrinsic defense mechanisms for reusing unfolding proteins in various cellular compartments.

Enhanced synthesis of HSP was detected in highly purified T cells during heat stress (febrile temperatures less than or equal to 41 °C), three major HSP with approximate molecular weights of 110, 90, and 75 were detected in these T cell populations. Enhanced HSP synthesis reflected augmented transcription of HSP genes which was contingent on the continued presence of hyperthermic stress (Ciavarra and Simeone 1990). Blake et al. (1990a, b) demonstrated HSP gene expression in rats exposed to heat shock. The patterns of heat stress induced HSP expression occur as a function of heat stress temperature and days of exposure,

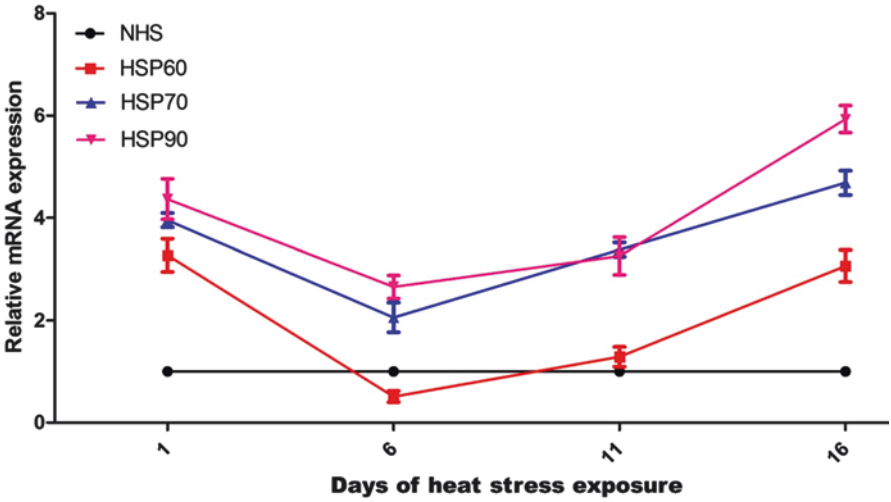

Fig. 2.1 Relative mRNA expression of HSP (HSP60, 70 & 90) during long term heat stress of 16 days in psychrometric chamber at 42 °C (6 hr./day); NHS-Non heat stressed animals, kept at ambient temperature

which is clearly an important component of the heat stress response and acclimation process (Dangi et al. 2014a). HSP expression pattern is at least two-peak phenomenon in animals i.e. primary window of HSP protection on first day followed by second window of protection on day 16 (Fig. 2.1) (Dangi et al. 2015; Dangi et al. 2016; Bharati et al. 2017a). HSP60, HSP70 and HSP90 play an important role during initial phase of heat stress acclimation whereas HSP105/110 joins this cascade at latter phase (Dangi et al. 2015).

2.1.4 Heat Shock Protein 70 (HSP70)

HSP70s are a family of ubiquitously expressed HSP. It is found in prokaryotes and eukaryotes (Tavaria et al. 1996; Yoshimune et al. 2002) and is mainly localized in the cytosol, mitochondria and endoplasmic reticulum and exhibit constitutive and inducible regulation. HSP70 gene family in bovines includes HSP70–1, HSP70–2, HSP70–3, and HSP70–4 gene. HSP70–1 is an intronless gene located on chromosome 23 and has 1926 nucleotides in goats (Gade et al. 2010). Under normal conditions, HSP70 functions as ATP dependent molecular chaperone that assist the folding of newly synthesized polypetides, the assembly of multi protein complexes and the transport of proteins across cellular membranes. Under stressful conditions, elevated HSP70 levels allow cells to cope with increased concentrations of unfolded or denatured proteins (Panjwani et al. 1999). Dangi et al. (2012) found significantly higher HSP70 mRNA expression during summer season than winter season in

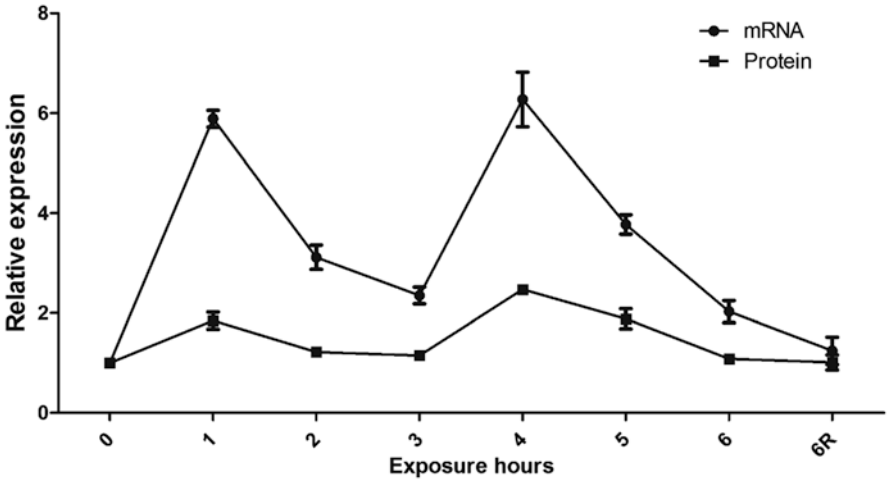

Fig. 2.2 Relative mRNA and protein expression of HSP70 in PBMC of goat at 41 °C (1–3 h), at 45 °C (4-6 h) and recovery period of six hours after exposure (6R)

tropical and temperate goats. HSP70 is the most temperature sensitive (Dangi et al. 2014). It increased with increase in THI in dairy cows (Liu et al. 2007). Dangi et al. (2014) exposed six Barbari goats to 41 °C temperature for 3 h and then to 45 °C for the next 3 h in psychrometric chamber and then recovery period of 6 h at moderate temperature. HSP70 transcript level was induced by heat stress, reaching the maximum (6.27- and 6.75-fold at 41 and 45 °C, respectively) at 1 h post-exposure. After that, the expression level declined gradually and achieved the pre-exposure level at recovery (6 h) (Fig. 2.2). Guerriero and Raynes (1990) reported that the critical threshold of thermal dose for HSP70 induction for bovines was 42°C for 3 h and the degree of its induction depends on the level and duration of exposure to stressors.

The increase usually is transient, but how long it persists is different in various cell types, ranging from hours, days, or weeks (Kiang and Tsokos 1998). HSP synthesis occurs at 5–15 °C above the optimal environmental temperature of that organism, depending on the organism's growth temperature range (Lindquist 1993). The response is rapid (usually within 2–5 minutes after heat shock), and the expression profile displays a temperature related dynamics, in which the levels of specific HSP change over the range of different heat shock temperatures (Lindquist 1993). Generally there is a transient increase in the synthesis of HSP at low level temperature elevation, with a more sustained response observed at higher temperatures, and this pattern of response has been consistently observed in numerous organisms (Lindquist 1993). Variation in HSP70 gene expression and polymorphisms has been positively correlated with variation in thermotolerance in *Drosophila melanogaster*, in *Caenorhabditis elegans*, in rodents and in humans. When the lymphocytes were exposed to different temperature for varying time duration, the level of HSP70 increased significantly (Patir and Upadhyay 2010). The prior incubation of

lymphocyte cells at 38°C for 48 h followed by thermal exposure of the same cells at 45°C for 3 h, resulted into an abrupt rise in the concentration of HSP70. The rise in the concentration of HSP70 could serve as a rapid protective mechanism against the applied thermal stress to maintain its cellular homeostasis. Buffalo lymphocyte cells heat shocked *in vitro* induced the level of HSP70, as observed in many of the livestock species, and higher the concentration of HSP70 in buffalo lymphocyte cells, higher the percentage of viable cells which might be a part of cellular protective physiological mechanism of responding to heat stress and thereby maintaining cellular homeostasis.

HSP70 are rapidly and abundantly up-regulated to protect cells, organs, and living organisms from damage in response to an array of stresses, including hyperthermia, inflammation, infection, chemicals such as ethanol, and exposure to numerous xenobiotics (Welch 1992). Elevated levels of HSP70, attained in transient or stable transfections, reduce or block caspase activation and suppress mitochondrial damage and nuclear fragmentation (Buzzard et al. 1998). Induction of HSP70 with arsenite reduced the cerebral ischemia, neural damage, and systemic hypotension, and increased the survival rate in rats exposed to heat stress (Yang et al. 1998). Intracellular heat shock protein 70 (iHSP70) accumulation improves heat tolerance through cytoprotective effects (Fehrenbach et al. 2000; Kregel 2002) such as the reduction of heat-induced cellular apoptosis (Selkirk et al. 2009). In addition to heat shock responses, iHSP70 also mediates cytoprotection to oxidative stress (Boshoff et al. 2000). Species that express high levels of iHSP can survive harsher environments than species that have lower levels of HSP (Tytell and Hooper 2001). Bharati et al. (2017a), exposed Tharparkar cattle at 42 °C temperature in psychrometric chamber for 6 h a day up to 23 days followed by 12 days of recovery period. Heat stress triggered the transcription and translation of HSP70 gene in PBMCs. The mRNA and protein expression increased ($P < 0.05$) on first day of heat challenge with the first peak on second day of exposure as compared to control and then decreased ($P < 0.05$) on 5th day of heat challenge to reach basal level. Thereafter, it gradually increased ($P < 0.05$) again to reach the second peak on 17th day of heat challenge and then decreased ($P < 0.05$) till the end of exposure period as compared to the control. HSP70 expression on the recovery period was comparable to control.

Heat shock induces HSP70 in the bovine lymphocytes (Guerriero and Raynes, 1990), bovine PBMCs (Kamwanja et al. 1994; Lacetera et al. 2006) and buffalo PBMCs (Patir and Upadhyay 2007). HSP70 levels in buffaloes at 42 °C and 70% RH were observed in serum: 200 fold increase and in lymphocytes: 2.5 fold increase compared with control animal under natural conditions (Mishra et al. 2010). The mRNA level of HSP70 in lymphocytes was increased with increase in THI; the mRNA level of HSP70 at high temperature was higher than others ($P < 0.01$) in dairy cows (Liu et al. 2010). The HSP70 concentration in Angus cattle increased from 0.07 to 0.25 ug/million cells when the temperature was enhanced from 38.5 °C to 42.4 °C whereas in Brahman cattle it increased from 0.07 to 0.26 ug/million cells when the temperature was enhanced from 38.5 °C to 42.0 °C (Kamwanja et al. 1994).

In vitro studies have shown that HSP70 is produced in heat stressed lung cells (Fargnoli et al. 1990), hepatocytes and liver (Heydari et al. 1995; Hall et al. 1999) and myocardium (Gray et al. 2000) thereby indicating that heat shock proteins provides protection from toxic effects of thermal stress. The HSP are released at specific temperatures and have critical temperatures for different livestock species viz.; 42°C (bovine and horse), 43°C (sheep) or 44°C (chicken). Proteins with molecular weights of 70 and 90 kDa were synthesized in all species. Additional proteins were found in bovine, ovine and chicken lymphocytes (Guerriero and Raynes 1990).

DiDomenico et al. (1982) found that once the cells are exposed to the temperature at which they are released, the cells are able to withstand any level of extremes of temperature. HSP not only enhance heat tolerance but also give capacity to resist hypoxia, ischemia and inflammation exposure to cellular toxins as heavy metals, endotoxins and reactive oxygen species, all imposing serious stress upon tissues and their composing cells (Snoeckx et al. 2001). Increase in HSP72 in persons with spinal cord injury after 12 weeks leg cycling was observed (Darryn et al. 2002). The mRNA and protein levels of UBI, E2, 20S, and GR were increased at 6 and 24 h post exercise (Willoughby et al. 2003). In abdominal muscle, polyubiquitin mRNA levels increased during both hypo- and hyper-osmotic stress (Jeffrey et al. 2002). It is not yet known if the same genes are involved in heat tolerance in *Bos indicus* and *Bos taurus* but it is clear that in both cases tolerance is observed at the cellular level (Hansen 2004).

HSP70 has a significant role in cell thermo-tolerance and animal survival (King et al. 2002). However, an increasing number of observations have indicated that they may be released into the extracellular space (eHSP70) having a wide variety of effects on other cells (Tytell 2005). eHSP are particularly interesting as potential danger signals due to their massive induction by sub-lethal and lethal stresses and their ability to bind to immune effecter cells (Broquet et al. 2003). The source of eHSP70 during heat stress has not been fully elucidated (Hom et al. 2012). However, studies suggest that the source may be from damaged intestinal cells (Shapiro et al. 1986).

2.1.4.1 Heat Shock Protein 90 (HSP90)

HSP90 is a molecular chaperone and is one of the most abundant proteins expressed in cells (Csermely et al. 1998). It is highly conserved and expressed in a variety of different organisms from bacteria to mammals including prokaryotic analogue htpG (high temperature protein G) (Chen et al. 2006). It has been identified in the cytosol, nucleus and endoplasmic reticulum, and is reported to exist in many Tissues (Kunisawa and Shastri 2006). Heat stress induced expression of HSP90 was found in heart, kidney and liver tissues peaked after a 2 h heat exposure and reduced to basal level after increased duration of heat stress. Recently *in vitro* studies also showed the increased expression of HSP90 in bovine PBMCs at 2 h post heat stress (Kishore et al. 2013). HSP90 expression was increased at 42°C in cattle (Mahato

2014) and during summer season in goats (Paul 2014). In unstressed cells, HSP90 plays a number of important roles, which include assisting in folding (Buchner 1999), intracellular transport, maintenance and degradation of proteins as well as facilitating cell signaling. It acts as a general protective chaperone (Miyata and Yahara 1992; Wiech et al. 1992). HSP90 also participates in many key processes in oncogenesis such as self-sufficiency in growth signals, stabilization of mutant proteins, angiogenesis and metastasis (Fontana et al. 2002; Calderwood et al. 2006; Whitesell and Lindquist 2005; Sato et al. 2000).

HSP90 is mostly cytosolic and is expressed at high levels (accounting for up to 1– 2% of total cellular protein content) even in unstressed conditions (Jakob and Buchner 1994). HSP90 functions primarily in the "final" maturation of proteins and the assembly of complex macromolecular structures and also functionally interacts with a much more select group of substrates, termed "client" proteins, which include many kinases and transcription factors (Wiech et al. 1992). HSP90 also functions in an ATP dependent cycle that dictates and is in turn influenced by a complex network of co-chaperone proteins. HSP90, like HSP70 and HSP60, binds ATP and undergoes a conformational change upon ATP binding needed to facilitate the refolding of denatured proteins (Galea-Lauri et al. 1996). HSP90, like HSP70, inhibited apoptosis as a result of a negative effect on Apaf-1 function. HSP90 directly binds Apaf-1 and inhibits its oligomerization and further recruitment of procaspase-9 (Pandey et al. 2000). HSP90 has also been shown to interact with and stabilize RIP-1 kinase, an antiapoptotic protein that interacts with death receptors and degradation of RIP-1 in the absence of HSP90 precludes activation of nuclear factor-kB (NFkB) mediated by tumor necrosis factor-α (TNF-α) and sensitizes cells to apoptosis (Lewis et al. 2000). In unstressed cells, HSP90 plays a number of important roles, which include assisting in folding (Wiech et al. 1992), intracellular transport, maintenance and degradation of proteins as well as facilitating cell signaling. It acts as a general protective chaperone (Miyata and Yahara 1992; Wiech et al. 1992). HSP90 also participates in many key processes in oncogenesis such as self-sufficiency in growth signals, stabilization of mutant proteins, angiogenesis and metastasis (Calderwood et al. 2007). Bharati et al. (2017b) reported that the HSP90 increased significantly ($P < 0.05$) on day 1 in comparison to control and maintained this level till day 19 and afterwards again increased significantly ($P < 0.05$) on day 23 of heat exposure of Tharparkar cattle at 42 °C for six hours each day in psychrometric chamber.

2.1.4.2 Peripheral Blood Mononuclear Cells as the Contributory Source of HSP

The source of HSP has not been properly elucidated. To know this, Bharati et al. (2017a) evaluated the temperature treatment (37 °C, 39 °C and 42 °C) *in vitro* on PBMCs culture system. The Peripheral Blood Mononuclear Cells (PBMCs) were suspended at a concentration of 2×10^6 live cells/ml in RPMI-1640 medium

containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml and kept for 30 minutes in media for stabilization. The cells were then plated out at 2×10^6 viable cells per well in three 12-well plate (one plate each for 37 °C, 39 °C and 42 °C) and incubated in a humidified CO_2 (5%) incubator. Subsequently, plates were taken out from each respective temperature after heat challenge of 1 h, 3 h, and 6 h. After incubation total RNA was extracted from PBMCs of different treatments and cell culture media were isolated by centrifuging it at 2500 rpm for 10 minutes. On the other hand, the basal sample representing the zero time point was harvested after completion of 30 min in stabilization phase which served as control. Cell culture media was stored at −80 °C for further analysis of HSP70 protein by ELISA. Presence of eHSP70 were quantified in cell culture media at 37 °C, 39 °C and 42 °C at all 1, 3 and 6 h of incubation. Across the temperature, concentration of eHSP70 increased with increase in temperature and maximum of eHSP70 was found at 42 °C. The relative mRNA expression of eHSP70 increased significantly ($P < 0.05$) at all 1, 3 and 6 h compared with control in time and temperature dependent manner. This shows that PBMCs are the contributory source of HSP in Tharparkar cattle.

2.1.4.3 Extracellular HSP (eHSP)

It has been demonstrated that HSP70 is present in the extracellular compartment in rats, humans, and cattle (Kristensen et al. 2004; Fleshner and Johnson 2005) and that plasma levels of HSP70 are increased by a variety of stressors (Johnson et al. 2005; Aneja et al. 2006). Release of extracellular HSP is associated with α1- and β-adrenergic receptor activation, and blocking these receptors abolishes exercise-induced increases in plasma HSP (Johnson et al. 2005). Secretion of HSP70 from mammalian cells has been demonstrated to occur through a nonclassical pathway involving lysosomal endosomes (Mambula and Calderwood 2006) because HSP70 does not contain a consensus secretory signal sequence. Necrotic cells and cells undergoing apoptosis are also possible sources of extracellular HSP (Mambula and Calderwood 2006). Once in the extracellular compartment, HSP70 has been shown to facilitate innate immunity (Campisi et al. 2003; Fleshner and Johnson 2005; Aneja et al. 2006). Exercise increases plasma HSP70 concentration and this increase can be attenuated by glucose ingestion and adaptation to exercise (Febbraio et al. 2004; Marshall et al. 2006). Although HSP70 is present in plasma of Holstein-Friesian cattle (Kristensen et al. 2004) there have been no studies evaluating the impact of various stressors on plasma concentrations and what, if any, role extracellular HSP has on the immune and or the endocrine system of cattle.

The HSP act cognitively in cellular and tissue homeostasis (Thompson et al. 2002; De Jong et al. 2009) and are released intra-cellularly and extra-cellularly in an inducible form in response to stress (Hightower and Guidon 1989; Hecker and McGarvey 2011). Among the HSP, the source of extracellular HSP70 (eHSP70) during heat stress has not been fully elucidated (Hom et al. 2012). However, studies suggest that the source may be from damaged intestinal cells (Shapiro et al. 1986).

eHSP70 has important functions in pro-inflammatory immune response (Pockley 2003); therefore, changes in eHSP70 may be an indication of cellular damage within the intestines (Doklandy et al. 2006). Soluble HSP70 can be detected in the peripheral circulation and in rapidly growing tumor tissues or stress-induced necrotic cells (Kotera et al. 2001). HSP could be detected extra-cellularly (Basu et al. 2000). Initially HSP were thought to be exclusively intracellular proteins that were only released into cellular environment upon cellular injury or necrosis, but not apoptosis and, as such, they were not generally regarded as PAMPs but considered to be "danger associated molecular patterns" (DAMPs) (Basu et al. 2000). DAMPs are molecules that serve as alternative ligands for PRRs but signal the presence of cellular damage, as distinct from the presence of pathogens, thus also activating the innate immune response (Bianchi 2007). However it is now apparent that HSP can be actively secreted into the extracellular environment by tumor cells or released from cells undergoing necrotic lysis in response to cytotoxic lymphocyte (CTL) or natural killer (NK) action, or viral infections (Feng et al. 2003; Mambula and Calderwood 2006; Merendino et al. 2010). HSP70 induces inflammation and regulates cytokine production in airway epithelium through a TLR4 and NFκB-dependent mechanism. eHSP70 provides a mechanism for controlling the excessive expansion of an inflammatory response. eHSP70 negatively regulates the production of pro-inflammatory cytokines of monocytes exposed to TLR agonists and contributes to dampen the inflammatory response. The addition of HSP70 to TLR-activated monocytes down-regulated TNF-α as well as IL6 levels. eHSP70 has been shown to activate monocytes, macrophages, and dendritic cells, and up-regulate the expression of pro-inflammatory cytokines (Mortaz et al. 2006). eHSP70-induced cytokine expression in these cells appears to be mediated through the TLR2 and TLR4 complexes in a CD-14 dependent fashion leading to the activation of NFκB as well as MAPKs (Satoh et al. 2006), whereas, iHSP70 has also been shown to play a role in the suppression of apoptosis by interfering with the apoptotic program (Gabai et al. 1998; Yaglom et al. 1999). HSP70, in its capacity as a chaperone, was involved in antigen uptake and presentation by macrophages and other members of the APC group (Suzue and Young 1996). Furthermore, antigens associated with HSP70 were found to elicit a more pronounced immune response than they did on their own (Barrios et al. 1994). It has been shown that eHSP70 can interact with APCs via a receptor and elicit the release of pro-inflammatory cytokines (Asea et al. 2000; Binder et al. 2000). TLRs also sense endogenous ligands released from cells undergoing unprogrammed necrotic death such as HSP70 (Vabulas et al. 2002).

To demonstrate the modulatory role of eHSP70 on the expression profile of HSP70, HSP90 genes in *in-vitro* cell culture system, isolated PBMCs were resuspended at a concentration of 2×10^6 live cells/ml in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and kept for 30 minutes in media for stabilization at different concentrations of HSP 70 protein at 0 μg/ml, 0.03 μg/ml, 0.3 μg/ml, 3 μg/ml (Bharati 2015). The cells were then plated out at 2×10^6 viable cells per well in four 12-well plate (3 well for each treatment on each plate) and incubated in a humidified CO_2 (5%) incubator at

Table 2.2 Mean ± SE of relative mRNA expression of iHSP70 during different time interval on treatment of PBMCs with different doses of eHSP70 at 37 °C in in-vitro cell culture system

		Time Interval				
	Dose of eHSP70	0 h	1 h	3 h	6 h	Mean for exposure hours
iHSP70	0.00 µg/ml	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aC}	1.07 ± 0.46^{aC}	1.09 ± 0.18^{aC}	1.05 ± 0.13
	0.03 µg/ml	1.00 ± 0.00^{bA}	2.73 ± 0.60^{abC}	3.73 ± 0.55^{aC}	4.68 ± 0.61^{aB}	3.71 ± 0.44
	0.30 µg/ml	1.00 ± 0.00^{bA}	5.24 ± 0.68^{aB}	6.78 ± 1.05^{aB}	6.52 ± 0.48^{aB}	6.18 ± 0.46
	3.00 µg/ml	1.00 ± 0.00^{cA}	7.67 ± 0.60^{bA}	10.10 ± 0.47^{aA}	8.47 ± 0.55^{abA}	8.74 ± 0.51
iHSP90	0.00 µg/ml	1.00 ± 0.00^{aA}	1.00 ± 0.00^{aB}	1.05 ± 0.13^{aC}	1.22 ± 0.27^{aC}	1.09 ± 0.09
	0.03 µg/ml	1.00 ± 0.00^{bA}	1.34 ± 0.26^{bB}	3.73 ± 0.42^{aB}	4.99 ± 0.55^{aB}	3.35 ± 0.70
	0.30 µg/ml	1.00 ± 0.00^{cA}	3.10 ± 0.85^{bcAB}	5.28 ± 0.72^{abAB}	7.39 ± 1.13^{aAB}	5.26 ± 0.88
	3.00 µg/ml	1.00 ± 0.00^{cA}	4.22 ± 0.61^{bA}	6.37 ± 1.01^{abA}	8.43 ± 1.00^{aA}	6.34 ± 0.87

Means marked with different small superscripts in same row and means marked with different capital superscripts in same column differ significantly ($P < 0.05$).

37 °C. Subsequently, one plate was taken out after 1, 3 and 6 hours for total RNA extraction. On the other hand, the basal sample representing the zero time point was harvested after completion of 30 minutes in stabilization phase without any HSP70 protein dose which served as control. One plate incubated was used for immunocytochemisty. qPCR was applied to investigate the relative mRNA expression. On administration of eHSP70 to the PBMCs cell culture system, the relative mRNA expression of iHSP70, HSP90 increased significantly ($P < 0.05$) at all 1, 3 and 6 hours compared with control except at 0.00 µg/ml dose in a dose and time dependent manner (Table 2.2). The immunoreactivity revealed that all genes under study were localized in cytoplasm as well as in nucleus.

2.1.5 Linkage of HSP with Stimulation of Innate Immune Response

High ambient temperature causes functional and metabolic alterations in cells and tissues including cells of immune system. Heat stress can modulate immune response and heat shock-induced HSP such as HSP70 can serve as endogenous adjuvant for induction of immunity by binding Toll Like Receptors (TLRs) (Takeda et al. 2003). TLRs are mostly expressed on APCs like monocytes, macrophages, dendritic cells

and B lymphocytes (Zhou et al. 2005; Yan et al. 2007). In addition of natural pathogenic ligands, TLR2 and TLR4 can also sense the endogenous ligands such as HSP60 (Ohashi et al. 2000), HSP70 and Gp96 (HSP90B1) (Vabulus et al. 2002) and trigger inflammatory responses. However intracellular HSP predominantly down-regulate the host inflammatory response, extracellular HSP may increase or decrease the host inflammatory response. The extracellular HSP are now included in a growing list of so called danger-associated molecular patterns (DAMPs), or alarmins (John et al. 2011). Numerous studies have implicated HSP in various aspects of the immunological response to antigens, leading to the proposal that these proteins carry out a "moonlighting" function as "chaperokines" (Asea 2006).

TLRs recognize microbial markers like protein, carbohydrate, lipid, nucleic acids and/ or their combinations in a unique, non-self-reactive manner to onset a complex signalling cascade to trigger a wide variety of transcription factors and inflammatory cytokines (Takeda and Akira 2003). The TLR mRNAs expression pattern have been demonstrated in mice (Pruett et al. 2004), humans (Zarember and Godowski 2002), cattle and sheep (Menzies and Ingham 2006), goat (Tirumurugaan et al. 2010) and buffalo (Vahanan et al. 2008). It has been well established that, TLRs play a vital role in activation of innate immune response during heat stress in mice (Dehbi et al. 2012) and goats (Paul et al. 2015). In human, some of the results demonstrated that heat shock could up-regulate TLR2 and TLR4 expression via p38 pathway in monocytes and increase the response of monocytes to LTA or LPS, suggesting heat shock might regulate host immune response through modulating TLR expression (Zhou et al. 2005). HSP are considered as an immunity-regulating dangerous signal due to their capability to induce cytokine production and DC maturation (Asea et al. 2000; Ohashi et al. 2000).

Amongst all TLRs, Toll like receptor 2 (TLR2) and Toll like receptor 4 (TLR4) recognize the damage-associated molecular patterns (DAMPs) to produce several pro-inflammatory cytokines to evoke the host immune response during heat stress (Janeway and Medzhitov 2002; Takeda and Akira 2003; Kawai and Akira 2010). Heat stress also modulates the expression dynamics of pro-inflammatory cytokines such as IL2 mRNA expression in chicken lymphocytes (Han et al. 2010), pig (Ju et al. 2014) and goat (Maurya et al. 2013) PBMCs and IL6 mRNA expression in livestock (Welc et al. 2012; Ju et al. 2014).

Expression of TLR2 and TLR4 in human monocytes increased quickly in response to heat shock, suggesting that heat shock might up-regulate TLR expression and thus influence the overall responses of immune cells to PAMPs. Recently, it has been found that some of the endogenous ligands mainly HSP could activate the TLR2 and TLR4 (Matzinger 2002). It is found that heat shock induces HSP70 in bovine lymphocytes (Guerriero and Raynes 1990), bovine PBMCs (Kamwanja et al. 1994; Lacetera et al. 2006) and buffalo PBMCs (Patir and Upadhyay 2007; Mishra et al. 2010). Thus a strong correlation may exist between HSP and TLR expression during heat stress.

The dynamic expression of TLRs and HSP after heat shock revealed that the induction of TLR4 and TLR2 was more rapid than that of HSP suggesting the up-regulation of TLRs in B cells by heat shock was independent of the induction of

HSP (Chen et al. 2005b). An alternative explanation for the activation of adaptive immunity was proposed by Matzinger (2002). The so-called 'danger theory' states that in addition, pAPCs can be activated by endogenous substances released by damaged or stressed tissue (Matzinger 2002). Members of the HSP family are candidate molecules that potentially signal tissue damage or cellular stress to the immune system (Wallin Robert et al. 2002). At least two TLR members (TLR2 and TLR4) appear to function as HSP receptors and can couple the binding of HSP70 to NFkB activity (Asea et al. 2000). TLR2 and TLR4 were shown to be activated in stress conditions and are able to act synergistically with other signaling pathways (van Heel et al. 2005). TLR4 is the major receptor for eHSP70 in the induction of chemokines and activation of DC both *in vitro* and *in vivo* (Chen et al. 2009). It has been suggested that HSP70-induced pro-inflammatory cytokine production is mediated via the MyD88/IL-1R-associated kinase/NFκB signal transduction pathway and that HSP70 uses both TLR2 and TLR4 to transduce its pro-inflammatory signal in a CD14-dependent fashion (Dybdahl et al. 2002). Heat shock stress might affect TLRs expression of immune cells and then modulate immune responsiveness to PAMPs to the full activation of innate and adaptive immune systems to fight against pathogenic microorganisms (Chen et al. 2005a). Heat shock could induce up-regulation of TLR4, TLR2, and TLR9 expression in human B cells (Chen et al. 2005b). Through binding to TLR2, TLR4, recombinant HSP70, HSP60 can stimulate monocytes and dendritic cells to release cytokines and increase their antigen-presenting capacity (Asea et al. 2000; Vabulas et al. 2002; Gobert et al. 2004). Results identified HSF as an element of IL6 transcriptional regulation in muscle (Welc et al. 2012, 2013). Inhibition of HSF1 attenuated hyperthermia-induced IL6 mRNA upregulation. TLR4-induced IL6 expression involves the activation of the JAK-STAT pathway (Kimura et al. 2005). TLR2 and TLR4 receptors with their cofactor CD14 and CD36 are reported to induce cytokine secretion in an HSP70 dependent manner (Asea et al. 2002). The complete coding sequence of TLR 1–10 in cattle has been studied (Menzies and Ingham 2006; Seabury et al. 2007). The natural ligands vary to the TLR family as per their specificity. TLR2 and TLR4 are best studied among all the TLRs for their wide range of role in recognition of PAMPs.

Bharati et al. (2017c) reported that TLR2 mRNA expression was increased and noted to be the highest ($P < 0.05$) on day 2 of heat exposure of Tharparkar cattle at 42 °C for 6 h each day in psychrometric chamber as compared to control and remained consistent up to day 4. Between day 10 to 17, it was significantly lower than first four days of heat exposure and further reduced to basal level between day 19 to 23 and recovery period. TLR4 mRNA expression was gradually increased to reach at peak on day 19 and thereafter finally declined during recovery period. IL2 mRNA expression was increased and found to be highest ($P < 0.05$) on day 5 of heat exposure as compared to control and then decreased ($P < 0.05$) to basal level on day 19 of heat exposure and again increased ($P < 0.05$) on day 23 and reduced to basal level during recovery period. IL6 mRNA expression was increased and reached the zenith ($P < 0.05$) on day 4 of heat exposure as compared to control. Then it was

observed to be decreased up to day 19 and thereafter again increased on day 23 and declined to basal level during recovery period.

The mRNA and protein expression of TLR 2/4 and IL 2/6 in the *in vivo* study might have been influenced by multiple factors. Therefore, Bharati et al. (2017c) further studied the heat stress challenge to isolated PBMCs in *in-vitro* cell to evaluate the mRNA expression of TLR 2/4 and IL 2/6 at different temperature-time combination under more controlled conditions at 39 °C and 42 °C for 1 h, 3 h and 6 h of incubation. The TLR2 mRNA expression increased ($P < 0.05$) at 6 h at 37 °C and at 1 h, 3 h and 6 h at 39 °C and 42 °C, compared to control. The TLR4 mRNA expression was higher at all temperature-time durations as compared to control. However, the highest ($P < 0.05$) expression was obtained at 42 °C with 6 h of incubation period. The IL2 mRNA expression increased ($P < 0.05$) at 3 h and 6 h at 37 °C and at 1 h, 3 h, 6 h at 39 °C and 42 °C, compared to control. The IL6 mRNA expression increased ($P < 0.05$) at 1 h, 3 h, 6 h at 39 °C compared to control. At 42 °C, 1 h incubation showed maximum increase ($P < 0.05$), which decreased at 6 h of incubation. All the aforementioned genes (TLR2, TLR4, IL2 and IL6) exhibited a differential expression dynamics during heat stress. TLR2 expression was down regulated after 2nd day while TLR4 expression remained consistent up to 19th day of heat stress exposure. Thus it can be suggested that TLR2 might have a role in acute immune response during short term heat stress acclimation while TLR4 might be essential to provide long term immune response to alleviate the deleterious effects of heat stress and confer thermo-tolerance in Tharparkar cattle.

Bharati et al. (2017c) reported a positive correlation existed between heat stress days and HSP70 ($P < 0.01$), HSP90, TLR2 ($P < 0.01$) and TLR4 ($P < 0.01$). There existed a negative correlation between HSP70 and all inflammatory cytokines. Given the response sensitivity and crosstalk between HSP, TLRs and cytokines during long term heat stress, heat stress response is linked to cellular pathways controlling cytokine production and innate immune system stimulation through HSP playing a central role (Bharati et al. 2017c).

2.1.6 HSP and Nitric Oxide Synthases (NOS)

NO is synthesized in multiple cell types from the amino acid L-arginine by three different isoenzymes, two of which are constitutive (neuronal and endothelial NOS), and one inducible (iNOS). NO is a reactive, gaseous and rapidly diffusible double-face molecule, which is due both to its short half life of approximately 3–5 seconds in the presence of oxygen, and to its reactivity towards a variety of molecular targets. Excessive NO may be damaging, but NO induced by eNOS could be beneficial to the host. In addition, eNOS and HSP70 may play a beneficial role (Lee et al. 2008). NO if present in the cells at low concentrations, have several important physiological roles, including vasodilatation, neurotransmission, learning and pathogen killing function (Lee et al. 2008). However, cellular response to heat shock involves

not only HSP but also several other bio-molecules (Kregel 2002). HSP and NOS are two families of proteins that seem to be particularly involved in the adaptive responses to the stress (Renis et al. 2003). NO produced by eNOS, is a key signaling molecule in vascular homeostasis (Ignarro 2002). A co-regulation exists between iNOS and HSP70 both in normal and in oxidative stress condition (Renis et al. 2003). eNOS-associated HSP90 may also serve as a scaffolding protein, facilitating the organization of additional associated regulatory proteins (Fleming et al. 2001). HSP90 over expression increases eNOS phosphorylation (Fontana et al. 2002).

Currently, most research works focus on the cellular and molecular mechanisms of array of heat stress response genes amongst which NOS family perceive a commendable attention in relation to thermal adaptation in livestock. Though NOS isoforms do not belong to HSP family still they are known to play a role in cellular homeostasis during thermal stress (Ali et al. 2012). Thus, the regulation of NOS isoforms is of utmost importance in maintenance of cellular homeostasis during the heat stress. The Nitric Oxide Synthases (NOS) family is comprised of neuronal nitric oxide synthase (nNOS), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) (Ali et al. 2012). NO, a gaseous free radical known for its wide range of biological activities, synthesized by the three NOS isoforms in the blood cells including PBMCs (Alderton et al. 2001; Yadav et al. 2016). NO regulates vascular hemostasis, hematopoiesis and immune response by modulating signaling, gene expression and the balance between proliferation and differentiation (Bogdan 2001; Coleman 2001; Nathan 1997). Optimum NOS expression produces NO which causes peripheral vasodilation during hyperthermia (Kellogg et al. 1998). NO can inhibit several intracellular enzymes and profoundly affect the cellular gene transcription machinery (Alderton et al. 2001).

Garcia-cardena et al. (1998) reported that HSP90 modulates NOS expression and signaling in endothelial cells and vascular system. It has also been reported that HSP90 enhances iNOS activity (Yoshida and Xia 2003). Moreover, HSP90 binds to eNOS and results a conformational change in eNOS and its activity (Pritchard et al. 2001). Thus, HSP90 serves as an allosteric enhancer of eNOS (Gratton et al. 2000).

Several studies proposed that HSP90 interacts with NOS isoforms and control their activity in cellular system. HSP90 interacts with iNOS and potentiates iNOS activity (Luo et al. 2011). HSP90 binds to the iNOS promoter region and induces iNOS transcription thereby acts an as endogenous enhancer of iNOS (Luo et al. 2011). Interestingly, Yoshida and Xia (2003) documented that the interaction between HSP90 with iNOS augments NO production as compared to the direct action of iNOS. Yoshida and Xia (2003) also revealed that, HSP90 seems to have a role in iNOS mediated cytotoxicity. The inhibition of HSP90 down regulates iNOS function (Yoshida and Xia 2003). Further, HSP90 inhibition markedly decrease NO production and prevents cell injury and ceases iNOS mediated cytotoxicity in macrophages (Yoshida and Xia 2003). However, recent studies investigated that iNOS activity can also be influenced by protein-protein interactions (Luo et al. 2011).

Previous studies demonstrated that, HSP90 modulates the eNOS activity and its function in vascular systems (Aschner et al. 2007). HSP90 maintains the proper

conformation of eNOS which favors NO production while its absence leads to a different conformation that favors O_2 generation (Xu et al. 2007). It has been seen that, association of HSP90 with eNOS triggers NO production in endothelial cells (Duval et al. 2007). Further, the interaction of HSP90 exempts eNOS from caveolin-3 which naturally keeps eNOS in an inhibited state (Gratton et al. 2000; Govers et al. 2002). HSP90 has also been involved in proper folding of NOS enzymes which helps in the insertion of heme into the immature protein (Billecke et al. 2002).

Bharati et al. (2017b) reported in Tharparkar cattle exposed to heat stress at 42 °C for 6 h per day in controlled environment of psychrometric chamber for 23 days that the HSP90 mRNA expression, which was significantly up-regulated ($P < 0.05$) on day 1 of heat exposure as compared to control period, remained consistent during short term of heat exposure (10 days), again increased and noticed greatest ($P < 0.05$) on day 23 of long term heat exposure and finally reduced to basal level during recovery period. The iNOS mRNA expression was found to be greatest ($P < 0.05$) on day 1 of heat exposure in comparison to control, gradually declined during short term heat exposure of 10 days, again increased on day 21 and 23 of long term heat exposure while revert back to basal level during recovery period. The eNOS mRNA expression was found to be increased up to day 3 of heat exposure, maintained at a constant level up to day 19 and noted highest ($P < 0.05$) on day 21 while reduced to basal level during recovery period. The mRNA expression of all aforementioned genes was further validated by western blot and the protein expression of all respective genes was almost similar to the level of transcripts pattern.

Their findings on iNOS and eNOS expression was comparable with earlier reports in goat (Yadav et al. 2016) where the expression of both genes were found to be significantly higher during summer season as compared to winter season. In a study conducted in rat, Sharma et al. (2000) also observed a higher expression of iNOS and eNOS in neurons during heat stress. Previous studies have also revealed the cytoprotective role of iNOS where iNOS prevents apoptosis thereby protect cardiomyocyte during heat stress (Arnaud et al. 2001; Mori 2007). Likewise, Lee et al. (2003) observed an increased level of NOS transcripts in hepatocytes during heat stress.

The *in vivo* expression pattern of HSP90 and NOS isoform might have been influenced by multiple factors. So, Bharati et al. (2017b) also evaluated the expression of all the aforementioned genes in different temperature-duration combinations in an *in vitro* PBMC cell culture model. The relative expression data of all the studied genes in cultured PBMCs indicate that iNOS and eNOS were highly up-regulated at highest temperature (42 °C) in all the incubation periods. Therefore, it can be inferred that the iNOS and eNOS could be involved in acquisition of thermo tolerance during long term heat exposure in Tharparkar cattle. Thus, the data of Bharti et al. (2017a) indicates that, HSP90 may possibly interact with NOS isoforms and the association between HSP90 with iNOS and eNOS could provide a better protection during long term heat exposure in Tharparkar cattle. Therefore it can be suggested that, a dynamic equilibrium between HSP90 and NOS isoforms might play a

crucial role to confer thermo tolerance during heat stress acclimation in Tharparkar cattle.

2.1.7 Oxidative Stress and HSP

Heat stress increases lipid peroxidation which is associated with production of large number of free radicals, capable of initiating peroxidation of polyunsaturated fatty acids. Heat stress may lead to increased production of transition metal ions (TMI) that can make electron donations to oxygen forming superoxide or H_2O_2 which is further reduced to an extremely reactive OH radical causing oxidative stress. Superoxide dismutase (SOD) in conjugation with catalase and glutathione peroxidase (GPx) scavenges both intracellular and extracellular superoxide radicals and prevents lipid peroxidation. Heat-induced oxidative stress plays a pivotal role in the induction of apoptotic cell death and antiapoptotic HSP (Gorman et al. 1999). Possible linkage between heat and oxidative stress has been found and oxidative stress strongly enhances heat sensitivity. *In vitro*, HSP can be induced by ROS (Marini et al. 1996) and by the RNS compound (Adrie et al. 2000). Supplementation of antistress vitamins such as vitamin C and E were found to be most effective in enhancing the levels of antioxidants and decreasing lipid peroxidation (Zaidi and Banu 2004).

Oxidative stress occurs due to excessive production and accumulation of reactive oxygen species such as superoxide (O_2-), hydrogen peroxide (H_2O_2), hydroxide radicals (OH-) and singlet oxygen ($1O_2$) etc. in the cells due to stress. Small amount of free radicals or reactive oxygen species (ROS) are produced during normal metabolism, which is also required in many biochemical processes. But excessive accumulation of ROS beyond certain limits may damage biological macromolecules i.e. lipids, proteins, carbohydrates and DNA. External factors such as heat, trauma, ultrasound, infections, radiations, toxins etc. can lead to increased free radicals and other ROS and may lead to oxidative stress. GPx reacts with peroxides and requires glutathione (GSH) as the reductive substance donating an electron. GSH reduces oxygen toxicity by preventing O_2^- formation.

Besides, increased expression of HSP, there is also overload of free radicals causing oxidative damage, during heat stress. A number of biochemical and physiological events associated with hyperthermia can potentially promote free radical formation. Mitochondria, one of the main sources of ROS in cells, undergo a temperature- dependent uncoupling during increases in temperature (Salo et al. 1991). Hyperthermia also increases the conversion of the enzyme xanthine dehydrogenase to the oxidase form, an important source of oxygen-derived free radicals. Glutathione, an electron donor for the antioxidant enzyme glutathione peroxidase (GPx), increases rapidly in cells exposed to elevated temperatures, while depletion of intracellular GSH significantly sensitizes cells to a thermal stress (Freeman et al. 1990a, b).

2.1.7.1 Antioxidants and HSP Expression

Plasma ascorbic acid concentrations were reduced in cyclic Black Bengal goats during heat stress (Vikash 2004). Supplementation of anti-stress vitamins such as vitamin C and E was found most effective in enhancing the levels of antioxidants and decreasing lipid peroxidation (Zaidi and Banu 2004). Vitamin C is a known antioxidant that protects the structural integrity of the biomolecules of the organisms. Dietary supplementation of vitamin C reduces the severity of the heat stress (Lauridsen et al. 1997) by enhancing the antioxidant capacity of reactive oxygen intermediates and free radicals scavenging enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione transferase against oxidative stress (Lobo et al. 2010; Blokhina et al. 2003; Birben et al. 2012) and by inhibiting glucocorticoid synthesis (Thaxton and Pardue 1984). Vitamin C induced an increase in baseline expression of HSP60 and HSP70 in *in vitro* culture of human lymphocyte (Khassaf et al. 2003). A vitamin C induced increase in baseline expression of HSP60 and HSP70 was previously reported, but there is controversy about the mechanism of this increase, and it has been suggested that vitamin C may exert prooxidant effects in some situations (Khassaf et al. 2003). Administration of the antioxidant ascorbic acid to heat stressed poultry attenuated the increase in HSP70 in heart tissue (Mahmoud et al. 2004).

The effects of dietary constituents on HSP have been studied in animal models. Kelly et al. (1996) showed that vitamin E deprivation for 16 wk. combined with exercise for 8 wk. induced HSP72 expression in female rats. Vitamin E is a potent lipid-soluble antioxidant, and its dietary supplementation has been reported to prevent oxidative injury (Winklhofer-Roob et al. 2003). Vitamin E depletion in rats caused a significant increase in HSP32 and HSP70 expressions by alveolar and liver cells, which returned almost normal after vitamin re-supplementation (Topbas et al. 2000). The possible modulation of the heat shock signal transduction pathway by vitamin E was previously reported in human skin fibroblasts (Calabrese et al. 2001). α-tocopherol (a common isoform of vitamin E) has been shown to attenuate the increase of HSP72 in leukocytes after treadmill running (Niess et al. 1996), whereas Khassaf et al. (2003) showed that supplementation with ascorbic acid (vitamin C) attenuates the exercise-induced increase of skeletal muscle HSP70 while at the same time increasing baseline levels of HSP72, superoxide dismutase, and catalase in human lymphocytes.

Betaine is a tri–methyl derivative of the amino acid, glycine, and is present in the cells of micro–organisms, plants and animals. In the past, it was most commonly thought of as either an osmolyte or a methyl donor (Kidd et al. 1997; Craig 2004). More recently, it has been described as a chemical chaperone, since it repairs denatured proteins and interacts with molecular chaperones, the heat shock proteins. In addition to these attributes, it has several other properties that make it a most attractive candidate for attenuating thermal tissue damage.

The presence of betaine (N-trimethylglycine) at concentrations of 2.5–25 mM decreased the induction of HSP70 gene expression caused by incubation of 3 T3 and SV-3 T3 cells in hypertonic (0.5 osM) medium (Petronini et al. 1993). Plasma

methionine of Alpine kids increased 10 min after injection of 0.1 or 0.2 g kg^{-1} BW of betaine (34.7 μM; $P < 0.05$) whereas 0.2 g kg^{-1} BW of betaine was required to increase plasma methionine in Angora kids (23.2 μM; $P < 0.10$) (Puchala et al. 1995). Low physiological concentrations of proline, glycerol, and especially glycine betaine activate the molecular chaperones, likely by assisting local folding in chaperone-bound polypeptides and stabilizing the native end product of the reaction (Diamant et al. 2001). Diamant et al. (2001) showed that mitochondrial protein was irreversibly inactivated by heat treatment at 44 °C within 15 minutes. Remarkably, addition of betaine to the medium fully protected the protein from denaturation over the entire 40-minute duration of the trial. The amount of betaine in *Escherichia coli* cells was also correlated with a reduction in heat induced protein aggregation. Cells normally respond to thermal insults by synthesizing heat shock proteins that restore the functionality of denatured proteins by refolding them and preventing them from aggregating. In the trial mentioned above, betaine increased the rate at which heat shock proteins refolded proteins by up to 50% and the rate at which proteins were disaggregated by 2.5 folds. Sheikh-Hamad et al. (1994) observed a threefold reduction in thermally induced heat shock protein expression in canine kidney cells that had been treated to accumulate betaine. They concluded that betaine had protected intracellular proteins to such an extent that the signal for heat shock protein induction was attenuated. Dietary supplementation of 2 g betaine, decreased rectal temperature (RT), respiratory rate (RR), heart rate (HR) and heat load in sheep (DiGiacomo et al. 2012).

2.1.7.1.1 Vitamin C, Vitamin E Plus Selenium and Betaine

Vitamin C and Vitamin E supplementation increased the antioxidants level in goat blood (Sivakumar et al. 2010) whereas heat stress lowers the concentrations of antioxidant vitamins in serum and tissues (Sahin and Kucuk 2003). Heat stress increases oxidative stress with lipid peroxidation and HSP expression (Ananthan et al. 1986; Mujahid et al. 2007; Tan et al. 2010). Vitamin E is a well known first line of defence against lipid peroxidation (Mcdowell 1989). When the concentration of antioxidant vitamins decreases, lipid peroxidation increases in the plasma and tissues, leading to damage in the integrity of cell membranes (Mcdowell 1989). Ascorbic acid and vitamin E supplementation decreased expression of HSP70 (Khassaf et al. 2003; Sahin et al. 2009; Dangi et al. 2015). Strong relationship exists between lipid oxidation and HSP70 synthesis in stressed cells (Mahmoud et al. 2004). Selenium (Se) is an essential trace mineral incorporated into the selenoenzymes such as glutathione peroxidase (GSHpx). GSHpx reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in the GSH/GSSG antioxidant system and protects cells from oxidative damage (Rivera et al. 2005). Selenium supplementation decreased the need for cellular protection attributed to stress

induced HSP70 (Rivera et al. 2005) and imparted resistance to oxidative stress associated with high temperature exposure.

In vitro studies using canine kidney cells show that betaine can inhibit HSP70 mRNA expression at high temperatures, possibly by stabilising cellular proteins and protecting them from heat stress induced denaturation (Sheikh-Hamad et al. 1994). Betaine could have protected intracellular proteins to such an extent that the signal for heat shock protein induction was attenuated (Sheikh–Hamad et al. 1994). In a similar study, Diamant et al. (2001) showed that mitochondrial protein was irreversibly inactivated by heat treatment at 44 °C within 15 minutes. Remarkably, addition of betaine to the medium fully protected the protein from denaturation over the entire 40–minute duration of the trial. The amount of betaine in Escherichia coli cells was also correlated with a reduction in heat–induced protein aggregation (Diamant et al. 2001). Cells normally respond to thermal insults by synthesizing HSP that restore the functionality of denatured proteins by refolding them and preventing them from aggregating. Betaine increase the rate at which HSP refolded proteins by up to 50% and the rate at which proteins were disaggregated by 2.5–fold (Diamant et al. 2001). The exclusion of betaine from the incubation medium of heat–exposed avian fibroblast cells, failed to elicit a HSP response (Petronini et al. 1993) which emphasizes an important point that the transporter, which enables cells to take up betaine is induced by extracellular hyperosmolarity, but not by heat (Sheikh–Hamad et al. 1994). Heat stress causes increased oxidative stress with increased production of lipid peroxidation and HSP expression (Ananthan et al. 1986; Mujahid et al. 2007; Tan et al. 2010). Oral pretreatment with betaine significantly ($P < 0.001$) inhibits the level of lipid peroxidation, corticosterone and significantly protects the enzymatic antioxidant defense mechanisms in restraint stress-induced animals and thus, betaine ameliorates the tissue and cellular effect of stress (Ganesan et al. 2011).

To demonstrate whether the antioxidants (vitamin C; vitamin E plus Selenium) and chemical chaperone (betaine) administration alter the HSP expression, (Dangi 2014) divided 30 goats into 5 groups (n = 6) such as NHS (Non heat stressed, gp1), HS (Heat stressed, gp2), HS + B (Heat stressed administered with betaine, gp3), HS + VC (Heat stressed administered with Vitamin C, gp4) and HS + VE + Se (Heat stressed administered with Vitamin E and Selenium, gp5). Except NHS group, other groups were exposed to repeated heat stress (42 °C) for 6 h for sixteen consecutive days in psychrometric chamber. HSP mean relative mRNA expression of 16 days heat stress exposure period (6 h at 42 °C/day) in five groups showed that it was more in HS group than others (Fig. 2.3). When groups compared between days, expression of all HSP showed a similar pattern as first peak on day 1, reached to basal level on sixth day and followed by second peak on day 16. The relative messenger RNA (mRNA) and protein expression of HSP60, HSP70, and HSP90 was observed highest ($P < 0.05$) in HS group, followed by antioxidant and betaine administered group on day 1 and 16, which signifies that antioxidants and betaine have dampening effect on HSP expression. HSP105/110 expression was highest

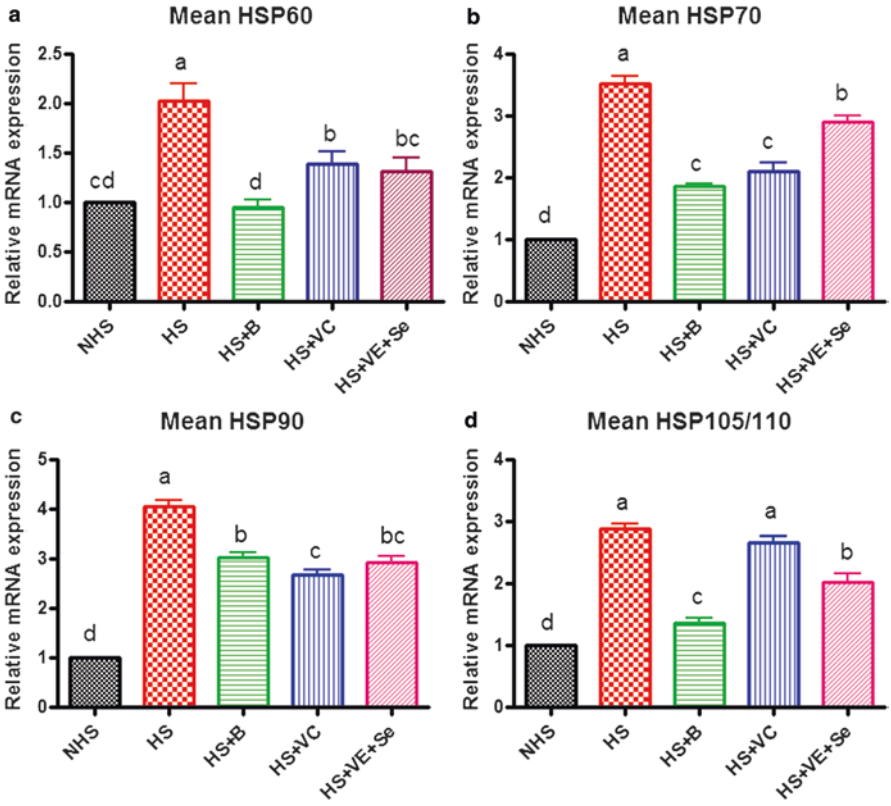

Fig. 2.3 A-3D HSP mean relative mRNA expression of 16 days heat stress exposure period (6 h at 42 °C/day) in five groups; Means bearing different superscripts (x–z) differ significantly ($P < 0.05$)

($P < 0.05$) on day 16. The results indicated that HSP expression pattern is at least two-peak phenomenon i.e. primary window of HSP protection on first day followed by second window of protection on day 16. HSP60, HSP70 and HSP90 play an important role during initial phase of heat stress acclimation whereas HSP105/110 joins this cascade at latter phase. Antioxidants may possibly attenuate the HSP expression by reducing the oxidative stress.

2.1.7.1.2 Melatonin

Melatonin is a much potent antioxidant as compared to vitamin C and E and plays prominent role in relieving heat stress by influencing cardiovascular system and evaporative heat loss (Harlow 1987). Melatonin, a tryptophan derivative, also known chemically as N-acetyl-5-methoxytryptamine from bovine pineal glands. It was first isolated and structurally identified in 1958 (Lerner et al. 1958). Subsequently,

melatonin was found to be a sleep promoter (Marczynski et al. 1964), a chemical signal of light and darkness as well as a regulator of photoperiod-dependent seasonal reproduction in some vertebrates. Originally, melatonin was believed to be synthesized exclusively in pineal gland of vertebrates. Studies have shown that many extrapineal tissues and organs have the capacity to synthesize melatonin. These include retina (Iuvone and Besharse 1983), ciliary body (Martin et al. 1992), lens (Abe et al. 1999), Harderian gland (Menendez et al. 1987) brain (Stefulj et al. 2001), thymus (Jimenez et al. 2005), airway epithelium (Kvetnoy et al. 1999) bone marrow (Tan et al. 1999) gut (Huether, G. 1993) ovary (Itoh et al. 1999), placenta (Iwasaki et al. 2005) lymphocytes (Carrillo et al. 2004) and skin (Fischer et al. 2006). It is estimated that melatonin generated from gut is in excess of two orders of magnitude greater than that produced by the pineal gland (Huether 1993). Highly elevated levels of melatonin, compared with those in serum, have been documented in the bone marrow, bile and third ventricular fluid of rats and sheep (Tan et al. 1999). Recently, Fischer et al. 2006 showed that the cultured keratinocytes contain unexpectedly high levels of intracellular melatonin. Potentially large quantity of extrapineal melatonin appears not to contribute significantly to the melatonin circadian rhythm in the circulation since surgical pinealectomy or chemical pinealectomy (constant light exposure), all markedly diminish the circulating melatonin levels in vertebrates (Vakkuri et al. 1985). In contrast, extrapineal melatonin synthesis is not subjected to light/dark regulation.

It could be speculated that the locally generated melatonin is consumed by the tissues in which it is produced as a protective mechanism of oxidative stress. Especially in the gut and skin, both of which are continuously exposed to the hostile outside environments such as food pollutants, bacteria, parasites, toxins and ultraviolet (UV) or other irradiation (Tan et al. 2007). The function of locally produced high levels of melatonin may be to help them cope with these stressors as antioxidant and anti-inflammatory agent. Melatonin supplementation has been shown to attenuate the retardation in performance as well as excretion of minerals caused by heat stress in broiler Japanese quail (Sahin et al. 2003). Harlow (1987) have shown role of melatonin in thermoregulation through influence on cardiovascular system and evaporative heat loss by increasing peripheral blood circulation. Melatonin also interacts with other hormones to alleviate heat stress possibly with thyroxine and successfully modify adrenal function to relieve thermal stress (Sejian and Srivastava 2009). Melatonin administration lowers core body temperature by 2–3 °C in mice (Charles et al. 1978). This observation correlates with the reduction in plasmal-thyroxine concentration observed in pinealectomized White Leghorn roosters, suggesting a possible link between melatonin and regulation of metabolic rate (Klandorf et al. 1978). Reduction in core body temperature with melatonin administration has also been found in chicks (Barchas et al. 1967), and several mammalian species (Kavaliers 1982), including humans (Strassman et al. 1991).

Some researchers have documented the increased HSP expression by melatonin stimulation, for example HSP60 in pancreatic cells (Bonior et al. 2005), HSP27 in HL- 60 cells (Cabrera et al. 2003) and others have shown decreased expression of

HSP70, HSP90 and HSP40 by melatonin administration in rat liver cells under oxidative stress (Catala et al. 2007). Melatonin reduces apoptosis in a number of experimental models, and also regulates the levels of mRNA for several proteins and exhibits endogenous antioxidant activities (Tan et al. 2003). Cabrera et al. (2003) have shown that melatonin enhances HSP27 mRNA level in response to heat stress. Sharma et al. (2013) also observed that the relative expression of HSP60 was increased manifold in melatonin treated goats at 40 °C exposure temperature. It confer protection to cell against heat stress by increasing expression of HSP60 as experimentally shown in heat stressed a AR42J pancreatic cells (Bonior et al. 2005). Collier et al. (2008) have reported that melatonin upregulates expression of HSP70 gene expression in bovine mammary epithelial cell during heat stress.

2.2 Conclusions

Gene expression is a fundamental process in all animals during heat stress. Rigorous understanding of heat shock responses of animal during short term and long term heat stress acclimation at the cellular and molecular level might upgrade the concept about how animals become tolerant to existing harsh environmental conditions in their niche. Above thermo neutral zone, stress gene networks receive both intra- and extracellular signals to coordinate cellular and whole-animal metabolism. Persisting stress alters physiological state referred to as acclimation via gene expression in response to endocrine signals or external signals. HSP expression pattern is at least biphasic or two-peak phenomenon. It requires a long term study to further study HSP peak phenomenon consistency. Some genes play important role during initial phase whereas others at later phase to smoothen the acclimation process to nullify deleterious effect of thermal stress by maintaining cellular homeostasis. In the acclimated state, metabolism is minimized to counteract the detrimental effects of increased thermal heat load. Nutritional manipulation can also be used to minimize thermal stress consequences. Considerable work is needed to define these networks and delineate opportunities for improving efficiency of domestic animals in warm environments.

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Chapter 3
Heat Shock Protein Expression and Implications in Spontaneous Animal Tumors: Veterinary and Comparative Aspects

Mariarita Romanucci and Leonardo Della Salda

Abstract Heat shock proteins (HSP) play a fundamental role in the maintenance of cellular homeostasis, under both physiological and stress conditions, by acting as molecular chaperones in protein folding, intracellular transport and degradation. HSP are also implicated in the hallmarks of cancer from proliferation, impaired apoptosis and sustained angiogenesis to invasion and metastasis. Altered HSP levels have been observed in a variety of human neoplasms and such abnormal expression may contribute to poor prognosis and drug resistance. Therefore, these molecular chaperones represent attractive targets for anti-cancer therapy. A growing number of studies in veterinary medicine have also demonstrated the presence of altered HSP expression in spontaneous animal tumors, especially canine cancer, and the study of carcinogenesis and the role of HSP in animal models represent an additional source of information for clinical cancer research. This chapter briefly reviews the current knowledge on HSP expression and implications in spontaneous animal neoplasms, and the advances in understanding of the therapeutic opportunities offered by HSP-based anti-cancer therapies in veterinary and comparative oncology.

Keywords Animal model · Cancer · Comparative oncology · Dog · Spontaneous tumors

Abbreviations

3-MA	3-methyladenine
APCs	Antigen-presenting cells
AR	Androgen receptor

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BPH	Benign prostatic hyperplasia
CMTs	Canine mammary tumors
CTVT	Canine transmissible venereal tumor
GA	Geldanamycin
GISTs	Gastrointestinal stromal tumors
gp96	glycoprotein 96
Grp78	Glucose-regulated protein 78
HO-1	Heme oxygenase-1
HSP	Heat shock proteins
HSPPC	HSP–peptide complexes
HUGO	Human Genome Organization
MCs	Mast cells
MCTs	Mast cell tumors
MHC	Major histocompatibility complex
OS	Overall survival
OSA	Osteosarcoma
PCa	Prostatic carcinoma
SCF	Stem cell factor
siRNA	small interfering RNA
TTP	Time to progression

3.1 Introduction

Heat shock proteins (HSP), also known as "stress proteins", are one of the most evolutionarily conserved classes of molecules, which are expressed by prokaryote and eukaryote organisms. HSP regulate protein biogenesis by assisting in the correct folding of newly formed polypeptides, oligomeric assembly and intracellular trafficking and are thus essential in the maintenance of cellular homeostasis. HSP also prevent inappropriate stress-induced protein aggregation by assisting in the repair of denatured proteins or by promoting their degradation. As a result of these functions, HSP have also been referred to as molecular chaperones (Nollen and Morimoto 2002; Macario and Conway de Macario 2007). HSP are classified mainly according to their molecular weight expressed in kDa and the best-studied HSP family members include Hsp27, Hsp40, Hsp60, Hsp70, Hsp90, Hsp110. However, the guidelines for the nomenclature of the human HSP families are currently based on the system recommended by the Human Genome Organization (HUGO) Gene Nomenclature Committee and use the Entrez Gene database from the National Center of Biotechnology Information (Kampinga et al. 2009). Each HSP family consists of several molecules, all sharing a similar primary structure and able to perform analogous functions in different subcellular compartments. HSP were so-called because their expression was originally discovered to be induced by heat shock (Ritossa 1962; Tissieres et al. 1974). However, since then, a wide variety of

environmental and metabolic factors including hypoxia, oxidative injury, glucose starvation, exposure to heavy metals, drugs or other chemical agents have been shown to elicit stress protein expression. Cellular stress response is a unique and essential defense mechanism put into act by the cell to cope with a wide range of harmful conditions. This response includes increased HSP synthesis, which has been detected in many pathophysiological conditions such as tissue injury and repair, hypertrophy, fever, inflammation, viral and bacterial infections (Morimoto 1998). In addition, HSP have been reported to be highly expressed in a wide variety of human cancers. In this respect, scientific studies focusing on the role of HSP in carcinogenesis have increased over time, demonstrating that HSP are actively involved in all phases of cancer from proliferation, impaired apoptosis and sustained angiogenesis to invasion and metastasis (Lianos et al. 2015; Wu et al. 2017). Likewise, abnormal HSP expression has been observed in various types of spontaneous animal neoplasms, especially canine tumors, suggesting a similar pattern of cancer development and progression. These comparable findings underline the relevance of spontaneous animal models in studies aimed at elucidating the multiple roles of HSP in carcinogenesis both in animals and humans (Romanucci et al. 2008; Della Salda and Romanucci 2012).

This chapter focuses on the current knowledge concerning HSP expression and implications in spontaneous animal neoplasms, and the advances in understanding of the therapeutic opportunities offered by HSP-based anti-cancer therapies in veterinary and comparative oncology.

3.1.1 Heat Shock Protein Expression in Spontaneous Animal Tumors

In veterinary medicine, the interest towards the roles of HSP in cancer has increased over time and most of the studies investigating HSP expression in spontaneous animal tumors were carried out on the canine model. As a matter of fact, several characteristics allow considering spontaneously occurring tumors in dogs as an attractive model for human cancer. In this respect, companion animals live in the same environment as humans and share similar environmental risk factors (Rowell et al. 2011). Tumor initiation and progression are also influenced by age, nutrition, sex, and reproductive status in both human and canine cancers (Di Cerbo et al. 2014; Fenger et al. 2014). Naturally occurring canine neoplasms also represent autochthonous tumor models which are believed to reproduce human tumors more closely than transplanted tumors, as they show orthotopic growth in an immunocompetent patient, tumor growth over long periods of time, inter-individual and intra-tumoral heterogeneity, tumor histology devoid of transplantation induced changes, metastasis via lymphatic and vascular vessels surrounding and within the primary tumor (Talmadge et al. 2007; Fenger et al. 2014). There is also a greater genetic homology between dogs and humans than between either species or the mouse (Kirkness et al.

2003; Switonski et al. 2004). In this respect, the identification of the canine genome sequence has provided further evidence of strong similarities with humans (Lindblad-Toh et al. 2005; Ostrander et al. 2006), as concerns various cancer-associated gene families and the presence of analogous genetic cancer-associated molecular abnormalities (Mueller et al. 2007; Olson 2007; Di Cerbo et al. 2014).

One of the first studies concerning HSP expression in canine neoplasms was carried out on canine transmissible venereal tumor (CTVT), in which high levels of Hsp60 and Hsp70 were detected and it was thought that these HSP could be considered potential markers for CTVT cells (Chu et al. 2001). However, a number of scientific studies over the years have shown high levels of expression of different HSP in a variety of canine tumors (Romanucci et al. 2008; Carrasco et al. 2011; Selvarajah et al. 2013; Asling et al. 2016; Romanucci et al. 2016, 2017), confirming that HSP expression cannot be relied upon for the identification of a specific tumor histological type (Ciocca and Calderwood 2005). Nevertheless, increased levels of Hsp60 were suggested to be involved in CTVT regression (Chu et al. 2001).

Thereafter, scientific studies in veterinary medicine have looked at the possible prognostic value of HSP expression, as well as at its potential for targeted therapy in canine cancer. As described in further detail below, most of the studies focused on canine osteosarcoma (OSA), mammary tumors (CMTs), mast cell tumors (MCTs) and prostate carcinoma (PCa), although single studies concerning HSP immunoexpression in canine gastric carcinoma (Carrasco et al. 2011), skin neoplasms (Romanucci et al. 2005; Bongiovanni et al. 2008), and peripheral nerve sheath tumors (Romanucci et al. 2013) are also available. Furthermore, as concerns spontaneous tumors in domestic species other than dog, the expression of various HSP has been investigated in normal urothelium, premalignant and malignant urothelial lesions of bovine urinary bladder, showing an altered pattern of HSP expression during the process of urothelial carcinogenesis. As a matter of fact, reduction of Hsp27 immunolabelling resulted to be significantly associated with increasing histological grade of malignancy, whereas increased immunoreactivity of Hsp60 and Hsp72 in both premalignant and malignant lesions indicated an early involvement of these proteins in neoplastic transformation of bovine urinary bladder mucosa (Romanucci et al. 2012b).

3.1.2 HSP and Canine Osteosarcoma (OSA)

OSA is the most common primary malignant tumor of bone in both dogs and children; however, the disease is significantly more common in dogs than in people. Canine OSA displays striking similarities in tumor biology and behavior, including metastatic propensity, responses to traditional treatment strategies such as surgery and chemotherapy, and dysregulation of several key molecular pathways, with its human counterpart. Therefore, canine patients offer a unique opportunity for autochthonous tumor studies (Mueller et al. 2007; Fenger et al. 2014).

A number of researches have been performed to investigate the expression and prognostic significance of HSP in human OSA (Trieb et al. 1998, 2000; Uozaki et al. 2000; Ozger et al. 2009; Moon et al. 2010; Liang et al. 2015). In addition, during the last decade, scientific studies have been focused on the potential usefulness of Hsp90 and Hsp70 inhibition as novel treatment approaches of human OSA (Bagatell et al. 2007; Gazitt et al. 2009; Fu et al. 2013; Gorska et al. 2013; Hu et al. 2015; Mori et al. 2015, 2017).

The expression and prognostic value of several members of the major HSP families have also been explored in canine OSA tissues (Romanucci et al. 2012a; Selvarajah et al. 2013). Hsp27, Hsp73 and Hsp90 showed a variably intense, cytoplasmic and nuclear immunoreactivity that was not associated with histological type or grade in canine OSA. On the other hand, a high percentage of Hsp72 immunostaining was significantly associated with high-grade canine OSA and a lack of immunolabelling was significantly correlated to a longer overall survival (OS) (Romanucci et al. 2012a). Thus, available data suggest an association between high Hsp70 immunodetection and poor prognosis in both human and canine OSA (Uozaki et al. 2000; Romanucci et al. 2012a). However, a correlation between the presence of Hsp72 expression and a better response to neoadjuvant chemotherapy was observed in human OSA patients (Trieb et al. 1998). In addition, a more recent study revealed that combination schedules with doxorubicin after pre-treatment with VER-155008, a small molecule inhibitor of Hsp70 and Glucose-regulated protein 78 (Grp78), were unable to significantly increase apoptosis or decrease cellular viability beyond that achieved by chemotherapy alone in canine OSA cell lines. Nevertheless, decreased cellular viability and clonogenic survival, as well as increased apoptosis were observed with single-agent VER-155008 treatment. These results were thus hypothesized to be due to the VER-induced upregulation of target proteins Hsp70 and Grp78, and/or other co-chaperone proteins occurring prior to the administration of chemotherapy (Asling et al. 2016). In this respect, a recent study demonstrated that small interfering RNA (siRNA)-mediated inhibition of Hsp70 expression in cultured human OSA cells was able to significantly enhance sensitivity to cisplatin (Mori et al. 2017). Therefore, these results indicate that enhanced antitumor effects may be obtained when chemotherapy is administered to OSA cells with Hsp70 expression inhibited. On the other hand, the only inhibition of HSP function by protein-binding inhibitors, such as the ATP-mimetic VER, prior to the administration of chemotherapy may induce a compensatory increase in the expression of target proteins which could explain the lack of improvement observed with combination treatments (Asling et al. 2016). Further studies are still necessary in order to determine the pathways involved in the potentially compensatory responses to HSP inhibition, which could provide useful information for optimizing combination schedules.

As far as Hsp90 is concerned, it was abundantly expressed in canine OSA tissues, suggesting the involvement of this chaperone in the pathogenesis of canine OSA, although no association seems to exist between its expression and tumor prognosis (Romanucci et al. 2012a), similarly to humans (Uozaki et al. 2000; Ozger et al. 2009). The widespread immunoexpression of this protein observed both in

primary tumors and in neoplastic emboli also underlines the possibility of using the canine OSA model for further testing of Hsp90-targeted cancer therapy (Romanucci et al. 2012a). In fact, Hsp90 is a specialized molecular chaperone, responsible for folding numerous oncogenic proteins and thus leading to a frequent 'addiction' to this Hsp by cancer cells (Trepel et al. 2010). Several studies have highlighted the possible effectiveness of Hsp90-binding agents in the targeted therapy of human OSA, especially in the context of childhood sarcomas (Bagatell et al. 2005, 2007; Gazitt et al. 2009; Hu et al. 2015; Heske et al. 2016). McCleese et al. (2009) have also demonstrated the selective cytotoxicity of the Hsp90 inhibitor STA-1474 for human and canine OSA cell lines. Hsp90 plays an important role in autophagy (Xu et al. 2011), and Hsp90 inhibition may induce autophagy through inhibition of mTOR (Palacios et al. 2010). Dysregulated autophagy is involved in several pathological processes, including cancer (Gottlieb and Carreira 2010). However, targeting autophagy in the context of anticancer treatments may be complex due to its dual role: activation of autophagy could either inhibit apoptosis or contribute to its induction, thus promoting cell survival or cell death, respectively (Eum and Lee 2011; Yang et al. 2011). In fact, even though autophagy modulation is considered a promising, novel mechanism for enhancing anticancer treatments (Duffy et al. 2015), it is still a matter of debate whether autophagy suppresses tumorigenesis or provides cancer cells with a rescue mechanism under unfavourable conditions (Grandér and Panaretakis 2010; Meschini et al. 2011). In this respect, a recent study showed that treatment with a combination of the Hsp90 inhibitor, geldanamycin (GA) and the autophagy inhibitor 3-methyladenine (3-MA) suppressed autophagy and induced apoptosis to a much extent in KTHOS human OSA cells than GA alone. It was considered that 3-MA suppressed a protective mechanism induced by Hsp90 inhibitor in KTHOS cells and induced apoptosis, thus suggesting that the combination of an Hsp90 inhibitor and an autophagy inhibitor may be an effective treatment for OSA (Mori et al. 2015). However, we recently investigated the effects of the GA derivative Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG) in D22 and D17 canine OSA cell lines, with the aim to explore the interplay between cell death, autophagy, and mitophagy, in light of the dual effect of autophagy in regulating cancer cell survival and death. The results of this study showed a simultaneous increase in apoptosis, autophagy, and mitophagy in D22 cell line, suggesting that 17-AAG-induced autophagy is likely to assist the proapoptotic activity of the drug in this type of cells. On the other hand, although 17-AAG treatment significantly increased autophagy and mitophagy in D17 cell line, low levels of apoptotic cell death were detectable, suggesting that 17-AAG-induced autophagy may play a protective role in these cells. These results thus revealed differential cell responses to drug-induced stress depending on tumor cell type, suggesting that pharmacological treatments based on proapoptotic chemotherapy in association with autophagy regulators would benefit from a predictive in vitro screening of the target cell type (Massimini et al. 2017). Additional investigations on the canine OSA model would be helpful in order to better understand the molecular mechanisms influencing the different responses of OSA cells to treatments and the crosstalk between autophagy and apoptosis.

A significantly increased expression of Hsp60 was also found in relation to shorter OS in canine OSA patients. In addition, gene silencing using siRNA approaches against Hsp60 promoted apoptosis and inhibited cell proliferation of canine OSA cell lines. These results therefore suggested an association between Hsp60 overexpression and poor prognosis in canine OSA, highlighting the need to evaluate Hsp60 as a new target for anticancer therapy, which could be tested in dogs and also used for human OSA studies (Selvarajah et al. 2013). In this respect, a GA-induced upregulation of Hsp60 gene expression was observed in human OSA cells, in association with a simultaneous loss of hyperacetylated Hsp60 mitochondrial protein pool, thus resulting in decreased viability and enhanced OSA cell death. On the basis of these results, not only Hsp90 inhibition, but also Hsp60 hyperacetylation was hypothesized to be associated with anticancer activity of GA (Gorska et al. 2013). Likewise, this study further underlines the potential utility of Hsp60 as a target for developing novel anticancer treatments to be tested in comparative oncology, also confirming that inhibition of multiple HSP could be a useful strategy to enhance the efficacy of HSP-targeting cancer therapy (Asling et al. 2016; Wu et al. 2017).

3.1.3 HSP and Canine Mammary Tumors (CMTs)

In veterinary medicine, mammary tumors constitute the most common malignant neoplasms in the bitch (Goldschmidt et al. 2017), showing wide pathological and clinical heterogeneity similar to the disease in humans. Similarities between human and canine mammary neoplasms on a molecular level also allow more significant comparative evaluations of the molecular mechanisms involved in carcinogenesis with respect to the classical rodent model. In this respect, a similar pattern of change in Hsp70, Hsp90 and apoptosis-associated proteins, such as Bcl-2, Bcl-XL, Bax, Caspases 3 and 8, was observed in both human and canine mammary tumors (CMTs), suggesting the existence of similar mechanisms to evade apoptosis in both humans and canines (Kumaraguruparan et al. 2006). Alterations of HSP expression, as well as their diagnostic, prognostic and therapeutic significance, have been extensively studied in human breast cancer (Ciocca and Calderwood 2005; Oskay Halacli et al. 2013; Davidson et al. 2016; Jagadish et al. 2016; Acun et al. 2017). Likewise, several studies have investigated the patterns of HSP expression and their implications in CMTs (Kumaraguruparan et al. 2006; Romanucci et al. 2006; Badowska-Kozakiewicz and Malicka 2012; Bongiovanni et al. 2015; Okada et al. 2015; Szczubiał et al. 2015), also in light of the potential usefulness of the animal model for the study of human breast cancer. The results of these studies indicated that multiple HSP, especially Hsp27, Hsp70 and Hsp90 (Kumaraguruparan et al. 2006; Romanucci et al. 2006; Badowska-Kozakiewicz and Malicka 2012), are involved in canine mammary gland carcinogenesis. In particular, a correlation between Hsp27 expression and tumor invasiveness in association with reduced OS was observed in CMTs (Romanucci et al. 2006). The detection of Hsp27, particularly in canine

mammary infiltrating neoplastic cells (Romanucci et al. 2006), gave support to the theory that Hsp27 overexpression may influence the invasive and metastatic potential of human breast cancer cells (Lemieux et al. 1997) by controlling their migration on laminin-5 (Rust et al. 1999). In Hsp27-overexpressing human breast cancer cells, an increased expression of matrix metalloproteinase 9 was also detected, appearing to be correlated with a down-regulated expression of the Src family tyrosine protein kinase Yes (Hansen et al. 2001). In this respect, Hsp27 overexpression was found to promote anchorage-independent growth, migration, and doxorubicin resistance in CMT cells, suggesting the involvement of this Hsp in epithelial-to-mesenchymal transition and anti-apoptotic signaling pathways in such cells (Lin et al. 2015). Similarly, Hsp27 overexpression was demonstrated to inhibit doxorubicin-induced apoptosis in human breast cancer cells (Hansen et al. 1999). The availability of two newly established, Hsp27-expressing CMT cell lines may represent useful cell models for basic researches in cancer biology and for the development of antitumor therapeutic agents to treat both human and canine mammary carcinomas (Hsiao et al. 2014).

3.1.4 HSP and Canine Mast Cell Tumors (MCTs)

Cutaneous MCTs are among the most common tumors in dogs, accounting for 16%–21% of all skin neoplasms (Blackwood et al. 2012). Previous studies have implicated the stem cell factor (SCF) receptor, KIT, in the aetiology of canine MCTs (London et al. 1996; Welle et al. 2008). KIT is a surface receptor tyrosine kinase, normally expressed on mast cells (MCs) and encoded by the proto-oncogene c-kit (Galli et al. 1994). Several studies have reported that about 15% to 40% of canine malignant MCTs carry c-kit mutations, particularly in exon 11, including various point mutations and tandem duplications in the juxtamembrane coding region, thus inducing constitutive signaling that promotes uncontrolled proliferation and survival (London et al. 1999; Ma et al. 1999; Downing et al. 2002; Zemke et al. 2002; Riva et al. 2005). Aberrations of c-Kit, including mutations, deletions, and duplications, have also been detected in various human malignancies, such as gastrointestinal stromal tumors (GISTs), mastocytosis, and mast cell leukemia (Lin et al. 2010; Kim et al. 2012). Therefore, as KIT-driven tumors, canine cutaneous MCTs are considered a unique spontaneous model to study KIT-dependent human malignancies (Patruno et al. 2014).

Hsp32, also known as heme oxygenase-1 (HO-1), has been identified as an important KIT-inducible survival factor and a potential therapeutic target in neoplastic human (Kondo et al. 2007) and canine (Hadzijusufovic et al. 2008) MCs. In this respect, targeting of this Hsp by pharmacologic inhibitors was found to be associated with reduced growth and induction of apoptosis of Hsp32-expressing primary neoplastic canine MCs isolated from surgical samples of grade I and grade II tumor cases (Hadzijusufovic et al. 2008). However, a recent study carried out by the authors demonstrated the immunohistochemical expression pattern of Hsp32 in

canine MCTs, revealing a tendency toward a loss of Hsp32 in poorly differentiated MCs (Romanucci et al. 2017). Therefore, further investigations on the efficacy of the pharmacologic Hsp32 inhibitors on neoplastic MCs isolated from aggressive canine MCTs are needed, in order to better define the usefulness of testing Hsp32 inhibitors in clinical trials enrolling canine patients with aggressive MCTs (Hadzijusufovic et al. 2008). Even though an association between Hsp32 and KIT immunohistochemical expression was not observed in canine MCTs (Romanucci et al. 2017), this could be due to the multiple signaling pathways contributing to Hsp32 expression in canine neoplastic MCs (Hadzijusufovic et al. 2008). It is also known that Hsp32 induction is not limited to KIT mutations in MCTs, since SCF-activated wild-type KIT may also promote Hsp32 expression in neoplastic MCs (Kondo et al. 2007).

Overexpression of Hsp90, a molecular chaperone for which KIT is a client protein, is predictive of adverse behavior in human GISTs (Kang et al. 2010). The inhibition of its function may also represent a promising therapeutic solution for KIT-driven diseases. In this respect, Hsp90 inhibitors have been demonstrated to be effective in down-regulating mutated, constitutively activated KIT protein in human MCs (Fumo et al. 2004). These inhibitors also exhibit activity against KIT-dependent and –independent canine malignant MCTs (Lin et al. 2008). Hsp90 is also one of the non-histone substrates of histone deacetylase inhibitors (HDACi) and the Hsp90-dependent pathway has been recognized as an important histone acetylation–independent anticancer mechanism for the HDACi-induced down-regulation of KIT in human GIST cell lines (Mühlenberg et al. 2009). A reduction in Hsp90 chaperone activity has also been suggested to contribute to the HDACi-induced loss of KIT expression observed in canine malignant MCs (Lin et al. 2010). However, a great variability in extent and distribution of Hsp90 immunohistochemical expression was detected among canine MCTs of different grades (Romanucci et al. 2017), which could be one of the reasons for the partial response of canine MCTs to treatments based on Hsp90 inhibition (London et al. 2011). In fact, the in vivo biologic activity of Hsp90 inhibitors may be difficult to predict on the basis of experimental models, since clinical responses may be dependent on addiction of the different tumors to Hsp90 and its client oncoproteins (Jhaveri et al. 2014).

3.1.5 HSP and Canine Prostate Carcinoma (PCa)

The canine prostate gland shares many morphological and functional similarities with its human counterpart. Apart from a few cases reported in domestic cats, human beings and dogs are the only species to spontaneously develop numerically important, clinically detectable prostate cancers, although the incidence of PCa is much lower in dogs than in men (Argyle 2009; LeRoy and Northrup 2009) and the precise cell of origin of canine PCa is unknown (Foster 2016). Canine PCa is usually diagnosed at an advanced stage and is not responsive to castration. The common occurrence of bone metastases, as well as the androgen-independence of canine

tumors, renders dogs with PCa a relevant model for studying advanced, hormone-refractory PCa in men (LeRoy and Northrup 2009).

A number of studies have focused on the implications of HSP during human prostate tumorigenesis and cancer progression, as well as on their correlation with prognosis in PCa patients (Cornford et al. 2000; Cappello et al. 2003; Glaessgen et al. 2008; Castilla et al. 2010; Ciocca et al. 2010; Lu et al. 2010; Miyake et al. 2006, 2010; Dong et al. 2013; Ma et al. 2014; Cordonnier et al. 2015; Nolan et al. 2017). The information obtained from these studies may provide the scientific basis for the design of antitumor treatments targeting HSP in combination with conventional therapies, with the aim to improve the survival of PCa patients in the next future (Hessenkemper and Baniahmad 2013; Chen et al. 2016; Chi et al. 2016; Kita et al. 2017).

In a recent study carried out by the authors, the immunoexpression patterns of Hsp60, Hsp72 and Hsp73 were compared between benign prostatic hyperplasia (BPH) and PCa in dogs, in order to investigate the possible involvement of these proteins in canine prostate carcinogenesis and provide useful information in comparative oncology. Results obtained showed a higher proportion of Hsp60-positive cells in PCa when compared with BPH, whereas Hsp73 immunostaining did not differ significantly between the two groups (Romanucci et al. 2016). Given the strict association between Hsp60 overexpression and androgen-independence in human PCa (Castilla et al. 2010), these data indicate the need to perform further studies concerning the role of Hsp60 in canine prostate carcinogenesis. In particular, further evaluation of Hsp60 expression in both primary and metastatic PCa would be interesting, in light of its potential use as a therapeutic target.

Given the transcriptional activities of the prostate basal cell marker p63 on the hsp70 gene (Ghioni et al. 2002; Wu et al. 2005), as well as the regulation of Hsp70 expression by androgen receptor (AR) signalling (Lu et al. 2010), the possible relationship between the expression of these three molecules in benign and malignant lesions of the canine prostate gland was also evaluated, in order to provide further insights into the role of putative androgen-independent basal/stem cell-like cells in prostate carcinogenesis. Hsp72 immunosignal was localized in nuclei of cells lying in close apposition to the basement membrane of acini forming a discontinuous layer along the basement membrane of hyperplastic lobules, whereas it was mainly absent or detectable in scattered cells in canine PCa cases (Romanucci et al. 2016). These results suggested a role for Hsp72 in prostatic basal cells, which lie in close contiguity to the basement membrane and are considered to represent or, at least, include the stem cell component of prostate epithelium (Signoretti et al. 2000; LeRoy and Northrup 2009). Nuclear pattern of Hsp72 immunoexpression also showed significant associations with nuclear scores of both AR and the nuclear protein p63 (Romanucci et al. 2016). Likewise, double immunofluorescent staining revealed Hsp72-AR, as well as Hsp72-p63 co-expressions in basal cell nuclei in both BPH and PCa cases (Romanucci et al. 2016). The expression similarities between Hsp72 and p63 could be related to the ability of various p63 isoforms to regulate hsp70 gene transcription (Ghioni et al. 2002; Wu et al. 2005). In addition, the significant association between Hsp72 and AR nuclear scores, as well as their

co-expression in basal cell nuclei, appears to be in line with the regulation of the expression of this Hsp by AR and its signaling observed in human PCa cells (Lu et al. 2010). These findings thus indicate the need for future studies aimed at characterizing the functional role of the combined expression of Hsp72, p63 and AR in benign and malignant lesions of the canine prostate gland. As most advanced human PCa are androgen-independent and the presence of androgen-independent malignant stem cells among the prostate basal cells is believed to play an important role in the development of the androgen-independent status (Feldman and Feldman 2001), such studies could provide useful information in comparative oncology to clarify the role of putative androgen-independent basal/stem cell-like epithelial cells in human and canine prostate carcinogenesis.

Increased Hsp90 expression has also been observed in canine PCa, suggesting the involvement of this molecule in carcinogenesis and tumor progression and supporting Hsp90 as a potential target for therapeutic intervention (Palmieri et al. 2014). However, despite promising preclinical data, different phase II trials evaluating the clinical activity of Hsp90 inhibitors in human patients with castration-resistant PCa revealed minimal clinical effects (Oh et al. 2011; Thakur et al. 2016), often associated with unacceptable toxicity (Oh et al. 2011). Therefore, in light of these results, the availability of a valid spontaneous animal model to further investigate the role of Hsp90 in prostate tumorigenesis, as well as its potential utility as a therapeutic target in PCa, appears particularly essential. The canine model would be also helpful to explore, in more detail, the emerging role of other HSP, such as Hsp27 (Cordonnier et al. 2015; Chi et al. 2016), in the progression of PCa to castration-resistant disease and the potential use of Hsp27 knockdown as a treatment strategy for patients with advanced disease.

3.1.6 HSP-Based Cancer Therapies in Veterinary Medicine: Current Knowledge and Future Perspectives

A great number of studies have demonstrated that HSP play essential roles in cancer development and progression, since their overexpression may elicit carcinogenesis by regulating cell growth, migration, invasion, angiogenesis and metastasis. HSP may also inhibit apoptosis and promote resistance to anticancer drugs. Therefore, these molecular chaperones represent attractive targets for anti-cancer therapy (Lianos et al. 2015; Wu et al. 2017). HSP70 and HSP90 family members are the best characterized molecules and most anticancer drugs in the field have been designed to target Grp78 and Hsp90. These drugs demonstrated efficacy against cancer cells in vitro and in animal xenograft models in vivo, and some are being tested in clinical trials. Since Hsp90 is a molecular chaperone that promotes the conformational maturation and stabilization of a wide variety of client oncoproteins, it has received much attention as a promising target for therapeutic intervention in cancer and is probably the best-studied among all the HSP proteins. A growing number of Hsp90

inhibitors have been designed and tested in clinical trials of a wide range of malignancies in the last decade, since they offer the possibility to simultaneously target multiple oncogenic pathways (Tatokoro et al. 2015; Wu et al. 2017). Notwithstanding this, to date, no Hsp90-targeting agent has been approved for use in the clinical practice.

Given that translation of a therapeutic into the clinic requires the use of animal models that parallel the biological, genetic, etiological, immunological and therapeutic properties of human cancer (Talmadge et al. 2007), the antitumor activity of Hsp90 inhibition has been investigated in a variety of canine tumor cells (Lin et al. 2008; McCleese et al. 2009; Clemente-Vicario et al. 2015; Massimini et al. 2017). Metabolism and excretion of BIIB021, an orally available, fully synthetic small-molecule Hsp90 inhibitor, currently under clinical evaluation (Yan et al. 2017), have also been analyzed in dogs (Xu et al. 2013). In addition, the safety and efficacy of the Hsp90 inhibitor STA-1474 have been explored in a Phase I trial involving dogs with spontaneous tumors in order to provide the groundwork for clinical studies in humans with cancer. This study provided information regarding gastrointestinal adverse events, evidence that hematologic and biochemical toxicities are unlikely to occur during treatment, demonstration of biologic activity in a pretreated patient population, and tolerability of drug administration over multiple cycles of therapy (London et al. 2011). In this respect, it is important to underline that STA-1474 is a prodrug of ganetespib, which is one of the most promising Hsp90 inhibitor due to its improved safety profile and superior efficacy when compared with other Hsp90-targeting agents (Jhaveri and Modi 2015). On the other hand, the availability of a valid spontaneous animal model that parallel the hallmarks of human cancer is particularly essential since, despite promising preclinical data, several earlier single-agent clinical trials of Hsp90 inhibitors failed owing to side effects or lack of anticancer activity (Oh et al. 2011; Thakur et al. 2016; Wu et al. 2017), while other trials only displayed partial responses in human patients (Wang et al. 2016), similarly to canine patients (London et al. 2011). Therefore, since the efficacy of HSP inhibitors as single-agents in cancer therapy appears to be limited, information obtainable from the canine tumor model would be helpful to optimize the use of these inhibitors in the context of combination schedules, in order to discover treatment strategies more likely to result in clinical effectiveness in both dogs and humans.

In the last decade, considerable attention has also been focused on active immunotherapy against cancer, which attempts to directly stimulate the host immune system in order to elicit tumor specific immunogenic responses capable of producing sustained antitumor activity while minimizing systemic toxicity. The immune system can be activated in different ways to recognize and kill cancer cells and a variety of immunotherapy-based strategies are continuing to evolve in both human and veterinary oncology (Park et al. 2016; Whiteside et al. 2016). In this respect, HSP, especially glycoprotein 96 (gp96) and Hsp70, have been found to play essential roles in eliciting antitumor immune responses by acting as carriers for tumor-derived antigenic peptides. The HSP–peptide complexes (HSPPC) that constitute HSP-based anticancer vaccines can efficiently cause cross-presentation of tumor antigens

by major histocompatibility complex (MHC) class I molecules, thus priming CD8+ cytotoxic T lymphocytes, as well as activating CD4+ T cells through the MHC class II pathway (Calderwood et al. 2016). Since vaccination with HSPPC purified from tumor, but not normal cells, are able to mediate specific and protective immunity against autologous tumors, it has been assumed that HSP-chaperoned peptides are tumor cell type specific, providing a fingerprint of the antigenic milieu of the cancer cell (Shevtsov and Multhoff 2016).

The HSPPC-based vaccine strategy is characterized by the isolation and purification of HSP from the resected tumor with subsequent reinfusion of the complex to allow the chaperone to interact with antigen-presenting cells (APCs), thus priming the lymphocytes with a wide variety of tumor-associated antigenic peptides. However, since HSP may not deliver efficient inflammatory signaling, a combination with agents with adjuvant activity or pro-inflammatory forms of therapy may be required to enhance vaccine efficacy (Calderwood et al. 2016). In this respect, tumor-derived HSPPC coupled with hydroxyapatite (HA) was proven to induce immunity against autologous tumors in a clinical trial on canine diffuse large B-cell lymphoma (DLBCL), demonstrating safety and efficacy in prolonging time to progression (TTP) and lymphoma-specific survival in canine patients when used in combination with dose-intense chemotherapy (Marconato et al. 2014). The efficacy and safety of an autologous HSPPC-vaccine plus chemotherapy as the primary treatment for dogs with advanced indolent B-cell lymphoma were also investigated, demonstrating the effectiveness of chemo-immunotherapy in prolonging TTP, with no increased toxicity (Marconato et al. 2015). These promising data thus provide the basis for further studies concerning HSP-based immunotherapies in canine cancer patients.

Gp96 and Hsp70–peptide-based vaccines derived from autologous tumor lysates have also been tested in a number of phase I to phase III clinical trials concerning several human tumor entities (Shevtsov and Multhoff 2016), especially melanoma (Tosti et al. 2014), showing the induction of immunological responses in a large number of patients treated with HSPPC. Notwithstanding this, since clinical responses were observed only in certain patient subgroups (Shevtsov and Multhoff 2016), further investigations are needed in order to optimize treatment schedules and to maximize the clinical efficacy of HSP-based vaccine strategies. In particular, multimodality cancer therapies based on HSP in combination with radio, chemo, and/or hyperthermia therapy could be treatment options to be tested in future clinical trials (Shevtsov and Multhoff 2016). For this purpose, the canine translational model may provide a powerful source of information to improve the current knowledge on HSP-based immunotherapy and to optimize combination treatments in both veterinary and comparative oncology.

3.2 Conclusions

HSP play a fundamental role in tumorigenesis and may represent therapeutic targets for cancer treatment, both in humans and animals. However, several crucial questions still need to be addressed before specific HSP inhibitors can be approved for clinical use. Firstly, HSP are essential for cellular survival of both cancer and normal cells, although tumor cells are considered to have a reliance on HSP, since they regulate the function of a number of cellular oncoproteins. Thus, since the optimal use of therapeutics targeting HSP depends on understanding the degree to which they participate in both neoplastic and normal cellular physiology, further studies are required to define a therapeutic window for the development of more-efficacious and less-toxic novel anticancer HSP inhibitors. The identification of HSP functions that are specific to cancer cells may also allow to avoid the organ-specific toxicities (e.g. liver or ocular toxicity) usually associated with the use of HSP inhibitors (Wu et al. 2017). In addition, the in vivo biologic activity of HSP inhibitors may not be as significant as predicted by experimental models, since responses may be dependent on addiction of the tumors to HSP and their client oncoproteins (Jhaveri et al. 2014). In this respect, different HSP family members may collaborate with each other in a signaling pathway and the inhibition of one HSP may induce the overexpression of other HSP which compensate for the inhibitory effect of a single inhibitor. Therefore, a combination of multiple HSP inhibitors, as well as optimized combination schedules with conventional chemotherapeutic agents, could be useful strategies to enhance the efficacy of HSP-targeting cancer therapy (Asling et al. 2016; Wu et al. 2017). Given that significant barriers to the effective use of HSP inhibitors in the clinical setting still exist, spontaneous tumor models that more closely reflect human cancers would be helpful to discover treatment strategies more likely to result in clinical benefit (London et al. 2011). Several questions concerning HSP-based immunotherapy in canine cancer are also still unanswered (e.g. if the elicited response is long-lasting, or if dogs would benefit from periodic booster vaccination, and at what intervals), although HSP-based vaccines remain an important area of development in veterinary and human oncology (Tosti et al. 2014; Marconato et al. 2015). Clarification of all these questions will allow not only better understanding the mechanisms of action of the different HSP in cancer cells, but also leading to the development of more-effective and safe HSP-based cancer therapies in veterinary and comparative oncology.

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Chapter 4
Heat Shock Protein as an Adjuvant in Veterinary Vaccines

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Abstract The conventional vaccines offer effective approaches to control several infectious diseases till date but are not always safe. In search for relatively safer vaccines, the developed new generation vaccines possess weak immunogenicity. Adjuvants are used to potentiate the immune responses induced by vaccine antigens and to achieve a desirable type of response. Heat shock proteins (HSP) are explored as adjuvants as well as therapeutic targets against tumors. HSP are ubiquitous group of proteins conserved in nature and induced to over express during various stressful conditions including heat. HSP are the single most abundant group of proteins inside the cell and during non-apoptotic cell death, they act as danger associated molecular patterns (DAMPs) and recognized by pattern recognition receptors (PRRs) present on the surface or inside the host cells resulting in stimulation of cells involved in immunity. The molecular mechanisms involved in the adjuvant activity of HSP are not fully understood. In this chapter, we will focus on some of the established functions of HSP and study its role as an adjuvant in veterinary vaccines.

Keywords Adjuvants · Heat shock proteins · Immune responses · Veterinary vaccines

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Abbreviations

APCs	Antigen presenting cells
CCR5	Chemokine receptor 5
CTL	Cytotoxic T lymphocyte
DAMPs	Danger associated molecular patterns
DCs	Dendritic cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor;
gp96	Glucose-regulated protein
HSP	Heat shock proteins
IFN-γ	Interferon gamma
LOX-1	Lectin-like oxidized low-density lipoprotein (LDL) receptor-1
LRP1	Lipoprotein receptor 1
MAP	*Mycobacterium avium paratuberculosis*
MCP-1	Monocyte chemo attractant protein-1
MHC	Major histocompatibility complex
MIP-1	Macrophage inflammatory protein-1
NOS	Nitric oxide synthetase
PAMPs	Pathogen associated molecular patterns
PRRs	Pattern recognition receptors
RANTES	Regulated upon activation normal T cell expressed and secreted
SREC-1	Scavenger receptor expressed by endothelial cells-1
TAP	Transporter associated with antigen processing proteins
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha

4.1 Introduction

Vaccination is the most successful tool for protection against many infectious diseases. The traditional or conventional method of vaccine preparation includes attenuation of live pathogenic organisms to diminish the pathogenicity while retaining its ability to reproduce, or killing the pathogen by controlled physical or chemical methods to retain the physical structure with abolition of viability, to induce a potent immunity. The conventional vaccines although induce both innate and adaptive immune responses and control the infectious pathogens, in most of the cases the vaccine alone is not sufficient for induction of full proof immunity. Further, the traditional vaccines are not always safe, as inefficient attenuation, mutation or inactivation could renounce the pathogenicity and could lead to vaccine associated diseases. With the advent of recombinant DNA technology, tailor made vaccines wherein purified protein antigens are used as vaccine candidates or recombinant DNA vaccines constructs encoding antigenic gene segments are found to be safer as compared to traditional vaccines. Vaccine efficiency could be improved by addition

of compounds known as adjuvants. The word "adjuvant" derived from the Latin word "adjuvare" was coined by a French veterinarian Gaston Ramon, while working with preparation of inactivated toxins isolated from diphtheria and tetanus organisms. He found that induction of abscess at the site of injection improved the antibody titer against vaccine (Ramon 1924). Since then several compounds like mineral salt (alum), emulsion (oil in water), cytokines, microbial compounds, microparticles and liposomes were used as adjuvants to increase the vaccine efficiency. The mechanism of action of various adjuvants is different and in all cases induction of innate immunity is mainly involved. A recent study suggests that a properly designed adjuvant could make a link between innate and adaptive immunities and successfully guide the later in a particular direction (Guy 2007). Microbial peptide components remain a favorable choice as they mimic the natural microorganisms and could be identified by host immune cells to induce innate immunity. The present chapter will explore the activity of one such component namely heat shock proteins and its specific role as an adjuvant in veterinary vaccinology.

4.2 Heat Shock Proteins and its Classification

Heat shock proteins (HSP) are ubiquitous group of proteins conserved in nature and are expressed in all prokaryotic and eukaryotic organisms and are the single most abundant group of proteins inside the cell. In 1962, during an experiment with fruit fly, *Drosophila melanogaster,* the heat shock response was identified accidentally wherein an inadvertent increase of temperature in the incubator housing led to change in the chromosomal puffing pattern of salivary glands resulting in over expression of a number of proteins at the similar time frame, that were later named as HSP (Ritossa 1962). In addition to heat, over expression of HSP could also be induced by wide variety of physiological and environmental stresses such as extreme pH, osmotic pressure, UV radiation, viral infection, hypoxia etc. (Craig 1985). HSP are classified into six major categories based on the biochemical functions and named according to their molecular weights as small HSP comprising of HSP10, HSP27, HSP40, HSP60, HSP70, HSP90 (gp96) and large HSP comprising of HSP104 and HSP110. Though various HSP show very little homology between the class, HSP from the similar category share high degree of homology between the species. During stress, they act as molecular chaperones, by preventing protein aggregation to keep the protein in their native form and also assisting with correct protein folding (HSP40, HSP60 and HSP70), unfolding the misfolded protein (HSP100) and transport of protein between cellular components including targeting the protein for ubiquitination (ubiquitin, HSP104). Majority of the HSP are distributed in the cytosol but some of them could reside in the endoplasmic reticulum as well (grp78, grp170, grp96) (Li and Srivastava 2004). Indeed, HSP are the single most abundant group of proteins inside the cell and they protect the cellular proteins. In addition to chaperonic activity, the extracellular appearance of HSP from non-programmed cell death and not from apoptosis act as danger signal, and

influence the immune system by stimulation of proinflammatory cytokines and maturation of dendritic cells (Basu et al. 2000). In many studies, HSP purified from tumor or virus infected cells have been found to induce antigen specific cytotoxic T cell (CTL) responses. The full length or a fragment of HSP gene segment (N or C terminal) fused with an antigenic gene in a DNA vaccine construct has been used as an adjuvant. HSP thus enhance antigen specific humoral as well as cell mediated immune responses against many cancer antigens and infectious diseases.

4.3 The Initial Work on Immunological Property of HSP

The immunological property of HSP was first shown while working with screening of anti-cancer molecules from tumor tissue (Srivastava et al. 1986). In this process, cancerous cells isolated from chemically induced fibrosarcoma tissue of mice were homogenized and separated by chromatography and each purified protein fraction was immunized in healthy mice and later challenged with cancer cells. Some of the purified protein fractions were found to prevent the growth of the cancer challenge by developing protective immunity. Interestingly, all the anti-cancer molecules isolated were heat shock proteins including HSP70, HSP90, Calreticulin, gp170 (Srivastava et al. 1986). In another study, HSP70 purified from healthy tissues did not protect mice from cancer challenge whereas HSP70 purified from mice tumor cells could induce protective immune response against tumor specific antigens and sequencing of HSP from both tumor and healthy cells showed no variation. It was found out that HSP isolated from cancer cells were associated with the antigenic fragment and the HSP70-antigen complex could elicit anti-cancer immunity and dissociation of the antigenic peptide from HSP70 could abrogate its anticancer property (Udono and Srivastava 1993). These studies indicated that immunity afforded was antigen specific and association of HSP with the peptide is essential for protective immunity. Association of cancer antigen with HSP90, gp96, calreticulin and gp170 corroborated the role of HSP with immunological property (Udono and Srivastava 1994). It was found that the immunity provided by HSP bound with the antigenic peptide preferentially was presented through MHC class I molecule and thus increased the antigen specific CD8+ T cell mediated responses whereas HSP or peptide alone could not elicit the peptide specific immune responses (Blachere et al. 1997). In one study, Mycobacterial HSP70 gene was fused with ovalbumin gene fragment and the purified fusion product showed ovalbumin specific CTL responses. Similarly HSP preparations from viruses, parasites or other infected cells could also carry antigenic epitopes and could elicit protective immune responses against specific pathogens.

4.4 Heat Shock Proteins as Adjuvants

Over the past two decades, considerable work on HSP have shown its adjuvant potential when combined with a subunit or DNA vaccine but the mechanism of immunological influences is not well established till date. Various evidences suggest the ability of HSP to bind with the antigen, protect the conjugated or unconjugated antigenic protein from intracellular proteases and its subsequent presentation to professional APCs to exert its immunological property. HSP bind to the nascent or denatured polypeptides and help in proper folding. HSP are immunogenic and act as adjuvants to stimulate the immunogenicity of heterologous polypeptides after binding to them.

The use of cellular or microbial derived HSP as adjuvants has a number of advantages:

1. Peptide binding affinity: The structural details show that HSP have two active domains namely a peptide binding and an ATP binding domain. The ability of HSP to bind with multiple proteins is by identifying short sequence amino acid motif mostly composed of aromatic or hydrophobic amino acid residues. HSP of low molecular weight (HSP70) bind to short peptides and higher class HSP (HSP110, gp170) bind to long peptides. Peptide binding sequence motifs vary between different peptides based on different HSP (Park et al. 2006; Binder 2014).
2. Pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs): Microbial HSP can act as PAMPs and could be recognized by pattern recognition receptors (PRRs) of host cell resulting in induction of proinflammatory cytokines, chemokines, maturation and migration of DCs to draining lymph nodes. HSP72 (a member of HSP70 family), HSP110 from necrotic cells induce production of pro-inflammatory cytokines such as TNF-α, IL-12 etc. and maturation of dendritic cells (DCs) and increase the cytolytic activity of NK cells (Manjili et al. 2005; Williams and Ireland 2007; Calderwood et al. 2016).
3. Receptor binding activity: HSP could interact with a number of receptors present on the surface of major antigen presenting cells (APCs), macrophage and dendritic cells (DCs) such as CD91, LOX1, SREC1, TLR2/4, Stabilin-1/FEEL-1 and could deliver the HSP bound antigenic complex to APCs (Murshid et al. 2016).
4. Presentation of antigen to professional APCs: Several studies have suggested that HSP could protect the peptide in the intracellular spaces and cytoplasm from the activity of protease enzymes, thereby reducing the dosage of antigen for immunization and increasing the concentration of antigen for presentation to APCs. It was reported that even picogram level of HSP-antigen complex could induce the immune system through DC-MHC-I mediated cross presentation to CTL and this response was 200–400 times higher than the antigen alone (Srivastava 2002). Glycoprotein 96 (gp96) complexed antigenic peptides can be presented by both MHC-I and MHC-II molecules but with a selective preference

to MHC-I mediated cross presentation and an increase in the CD8+ T cell effector functions (Doody et al. 2004). The reconstitution of exogenous soluble protein ovalbumin (OVA) with HSP90, HSP90-OVA complex protected the antigen and preferentially increased the cross presentation of antigen by DC mediated MHC-I molecule and induced CD8+ T cell responses (Oura et al. 2011).

4.5 Mechanism of Action

Many studies have demonstrated that HSP90, HSP70, gp96, Calreticulin bound-peptide complex could synergize the innate and adaptive immune responses in a much better way as compared to the peptide or HSP alone. The mechanisms by which HSP influence the immune responses are not fully established, but it was found that HSP-bound peptide interact with a number of cell surface receptors expressed on APCs like DCs and macrophages and increase the expression of various cytokines, chemokines resulting in an enhanced immune response.

The first reported surface receptor for HSP is CD91, which is known as low density lipoprotein receptor 1(LRP1). CD91 receptor is distributed in all the cells of the body with a lesser number found in DCs, which is primarily responsible for cross presentation of antibody through MHC class I molecule. The HSP-antigen could also interact with TLR4 and TLR2 and activate various pro-inflammatory cytokines like IL-6, TNF-α, GM-CSF and IL-12. However, there is no direct evidence for the association of HSP70 with TLR2 and TLR4.

Extracellular HSP bound peptide is presented to the T cells via the MHC-I pathway. Various scavenger receptors which are primarily responsible for oxidized low density lipoprotein also recognize the HSP peptide complex. HSP-antigen complex might interact with the surface receptor SREC I and Lox-1 present on the APCs and get internalized into the cytoplasm as a complex (Murshid et al. 2015). Following entry of the exogenous HSP-peptide complex, the peptide is released from the HSP and is processed into smaller peptide in the endosome or in the cytosol by proteasome. The processed antigenic peptide could be transported to endoplasmic reticulum by the transporter associated with antigen processing proteins (TAP1 and TAP2), followed by presentation with MHC-I molecule of DCs and prime the naïve CD8+ cytotoxic T lymphocytes, which is known as cross presentation. Alternatively, processed antigenic fragments could also be loaded with MHC-II molecule as classical antigen presenting pathway and present the antigen to naïve CD4+ T lymphocytes. Following binding of HSP peptide complex with DC cells, it migrates towards the draining lymph node and matures by enhancing the expression of co-stimulatory molecule on the surface, which is essential for activation of naive T cells.

4.6 Effects of HSP on Innate Immune Response

The entire pathogenic organisms have certain conserved molecular structure, known as PAMPs, which could be recognized by broad class of receptors present on the surface or inside the host cells, known as pattern recognition receptors (PRRs). The interaction between PAMPs and PRRs plays an initial role in induction of immunity. HSP can be actively secreted into the extracellular environment from tumor cells or cells undergoing necrosis and act as DAMPs, which can serve as alternative ligands for PRRs thus activating innate immune responses. HSP60, HSP70 and HSP90 (gp96) family members stimulate the secretion of cytokines and chemokines from innate immune cells and causes co-stimulatory molecular expression and activation of APCs. HSP independent of antigenic association could activate downstream signal transduction cascade to promote DCs and macrophages for the secretion of proinflammatory cytokines like TNF-α, IL-1β, and IL6. HSP70, HSP90, gp96 bound antigenic complex could interact with macrophages *in vitro* and upregulate surface expression of CD86 and proinflammatory cytokines TNF-α, IL-12, IL-1β, GM-CSF and IL-6. It can also stimulate the expression of chemokines such as MIP1, MCP1 and RANTES. HSP peptide complex induces nitric oxide synthetase (NOS) in macrophages and DCs leading to increased production of nitric oxide. It helps in maturation by expression of MHC-II and CD40, CD86 molecules and migration of DCs to the draining lymph node which is very important for priming the naïve T lymphocytes and generation of antigen specific adaptive immunity. Mycobacterial HSP70 functions as a PRR ligand and signal through a chemokine receptor 5 (CCR5) expressed in various cell types including DCs, macrophages, and memory T cells, which is important for immune responses. The naturally occurring CCR5 ligand promotes the recruitment of immune cells towards the site of inflammation following binding. Furthermore, CCR5 binding promotes the crosstalk between DCs and CD4 + T cells and enhance antigen specific T cells, thereby promoting adaptive immune responses (Floto et al. 2006).

4.7 Effect of HSP on Adaptive Immune Response

In addition to initiation of potent innate immune responses, HSP also stimulate adaptive immune responses. HSP conjugated vaccines cause maturation of DCs by expressing CD80/86. Activated DCs interact with the naïve T cells by interaction of CD28 on their surface. In addition to interaction of co-stimulatory molecules, cytokine production by the DCs is also necessary for the activation of T cells. Extracellular HSP are recognized by sentinel cells and mediate the immune responses resulting in initiation of specific immune responses or enhancement of pre-existing inflammatory mediators.

HSP act differently depending upon the location. Inside the cell, it acts as a chaperone to bind with the nascent peptide and prevents it from misfolding, but

extracellularly it acts as a danger signal. The structural dynamics in peptide binding ability of HSP have been studied in detail in HSP70 and HSP90. The HSP70 has two structural domains namely a 44 kDa N-terminal domain with ATPase activity that interact with a co-chaperone DnaJ and a 27 kDa C-terminal domain with peptide binding domain and a helical lid structure. Further, a linker is present between the two domains.

One of the major concerns with the use of HSP as an adjuvant is the conserved nature of sequence with the same class of HSP among different species, which could be minimized by selection of fragments that have less homology rather than whole fragment. The C-terminal domain of *Mycobacterium* HSP70 has much variation and share less homology between the species. The use of C-terminal fragment increased the DNA vaccine potency against hepatitis B virus (HBV) as compared to an N-terminal fragment based DNA vaccine or DNA vaccine containing HBV antigenic fragment alone (Li et al. 2006).

4.8 Heat Shock Proteins in Veterinary Vaccines

HSP70 is elicited in several pathogens that include *Mycobacterium tuberculosis* HSP70 (Bonorino et al. 1998; Aosai et al. 2002), *Leishmania infantum* HSP70 (Rico et al. 2002) and *Toxoplasma gondii* HSP70 (Aosai et al. 2002). The 70-kDa HSP of schistosomes play an important role in stimulating high levels of innate and adaptive immune responses. The Sj22.6 is a tegumental protein which is expressed during the lung-stage schistosomulum of *S. japonicum*. Immunization with Sj22.6 protein confers partial protection and increased levels of IgG. Recombinant SjHSP70 (rSjHSP70) induces humoral and cellular immune responses in BALB/c and C57BL/6 mice (Duan et al. 2015). Surprisingly, the rSjHSP70 alone could induce the highest protective response against *S. japonicum* cercarial challenge when compared with a combination of rSj22.6 and rSjHSP70 and rSj22.6 alone, in BALB/c and C57BL/6 mouse strains indicating that the rSjHSP70 protein acted as an adjuvant. Immunization with rSjHSP70 or the combination of rSj22.6 and rSjHSP70 resulted in a mixed Th1/Th2-type antibody response in BALB/c mice and a Th2-type antibody response in C57BL/6 mice. The efficacy of the heat shock protein was better than the ISA206 adjuvant in promoting the production of both pro-inflammatory (IFN-γ, TNF-α, IL-6, and IL-17A) and Th2/regulatory (IL-4, IL-10) cytokines in the C57BL/6 mice.

Besides having adjuvant property, the HSP also help in immunomodulation and results in stimulating cytokine-mediated immune responses (Ebrahimi and Tebianian 2010). A recombinant HSP from *Mycoplasma hyopneumoniae*, the heat shock protein P42 (rP42), is responsible for sero-conversion in a mouse model (Simionatto et al. 2012; Galli et al. 2012) with the antibodies binding specifically to the HSP of *M. hyopneumoniae* and thereby inhibiting the cell growth (Chen et al. 2003). The rP42 stimulated both the arms of immune responses when used along with a conventional whole-cell vaccine against enzootic pneumonia in piglets. The rP42 in

oil-based adjuvant was also able to produce a humoral and cellular immune responses in *M. hyopneumoniae*. In addition, the rP42 antigen stimulated a significant expression of anti-inflammatory cytokine IL-10. Thus, it was suggested that rP42 emulsified in an oil-based adjuvant holds a promising recombinant subunit vaccine candidate against enzootic pnenumonia (Jorge et al. 2014).

In another study, HSP70 derived from *Toxoplasma gondii* could induce the dendritic cell maturation and resulted in IL-12 responses (Kang et al. 2004). HSP60 acted as a ligand on *Histoplasma capsulatum*, a facultative intracellular fungal pathogen and mediated binding to CD18 receptors on human macrophages (Long et al. 2003). Development of recombinant HSP60 as a subunit vaccine and its subsequent immunization in mice resulted in protection from *H. capsulatum* challenge (Scheckelhoff and Deepe 2002).

HSP based vaccines had a significant role in different models of experimental tuberculosis. Th1 responses could be induced when mice were immunized with BCG peptides complexed to HSP that resulted in protection against murine pulmonary tuberculosis (Colaco et al. 2004). A DNA vaccine generated based on the HSP65 *Mycobacterium leprae* gene conferred protection and acted as a potent prophylactic and therapeutic vaccine candidate in a mouse tuberculosis model, stimulating CD8+ T cell activation, IFN-γ, TNF-α and reduction of lung injury (Bonato et al. 2004). Various studies have showed that *Mycobacterium aviumparatuberculosis* (MAP) HSP70 could be an effective subunit vaccine candidate against MAP (Koets et al. 2006; Uto et al. 2011; Yuan et al. 2014). The vaccine acted by stimulating the dendritic cells resulting in a Th1 immune response. Interestingly, HSP65 and HSP70 showed differential expression in different stages of paratuberculosis (Koets et al. 2001). Moreover, the anti-HSP70 antibodies produced against MAP-HSP70 could be used in improved diagnostic, screening of MAP in animal tissues and pathogenesis studies of MAP (Okuni et al. 2017) (Table 4.1).

The antiviral activities like antibody-dependant cellular cytotoxicity, NK cell activity and CTL activities are increased when the HSP bind to viral complexes (Brenner and Wainberg 2001). The chemokine CCR5, a major co-receptor of CD4 glycoprotein mediates cellular entry of CCR5 strains of HIV-1 or simian immunodeficiency virus (SIV). For example, the HSP70 linked CCR5 peptides, SIV gp l20 and p27 when immunized into macaques stimulated the CC chemokines and antibodies. Thus, the CCR5 was down modulated, increased the immune responses and conferred protection against SIV challenge (Bogers et al. 2004a). This vaccine was also effective in controlling mucosal SIV infection (Bogers et al. 2004b).

The mycobacterial or mouse HSP70 genes have been linked with human papillomavirus (HPV) and malaria parasite antigens (Cheng et al. 2001; Udono et al. 2001). Immunization of the CTL epitopes derived from SV40, herpes simplex virus-2, influenza virus, vesicular stomatitis virus, or lymphocytic choriomeningitis virus linked with gp96 or HSP70 resulted in virus-specific CTLs or protective immunity (Srivastava 2002).

Trichinellosis is a widespread zoonosis and a constant economic threat to pig industry and food safety (Gajadhar et al. 2009; Yang et al. 2010). Vaccination with the recombinant *Trichinella spiralis* HSP70 (rTS-HSP70) protein resulted in 37%

Table 4.1 List of heat shock proteins used in veterinary vaccines and cancer immunotherapy

HSP	Function	References
Mycobacterium avium paratuberculosis (MAP) HSP70	Act as an effective adjuvant in a subunit vaccine candidate	Koets et al. (2006); Uto et al. (2011); Yuan et al. (2014).
HSP70 linked CCR5 peptides, SIV gp l20 and p27	Elicit immune responses against mucosal SIV infection, Increased immune responses through down regulation of CCR5 chemokine	Bogers et al. (2004a); Bogers et al. (2004b).
Mycobacterial or mouse HSP70 gene linked with human papillomavirus (HPV) and malaria parasite antigens	Provide protective immunity through virus-specific CTLs	Cheng et al. (2001); Udono et al. (2001).
Trichinella spiralis HSP70 (rTS-HSP70) protein	Induced a strong systemic Th1/Th2 immune response and activated the dendritic cells	Wang et al. (2009).
Immunization of chickens with *E. tenella* antigen microneme protein and EtHSP70	Reduces the faecal oocyst shedding, enhanced body weight gain and elicited higher antibody responses against avian coccidiosis.	Zhang et al. (2012); Mun et al. (2003); Qazi et al. (2005).
Salmomnella Typhi and salmonella Typhimurium with recombinant GroEL (rGroEL) or HSP60-IL2	Increased both humoral and cell mediated immunity	Wolk and Sabat (2006)
HSP27, HSP40, HSP60, HSP70, HSP90	Act as biomarkers for cancer diagnosis, in assessing the disease progression and a smart drug conjugate for therapeutic purpose for targeting cancer cells	Wu et al. (2017)

reduction in muscle larvae load against *T. spiralis* larval challenge (Wang et al. 2009). The vaccine induced a strong systemic Th1/Th2 immune response and conferred protection by activating the dendritic cells. Similar approaches based on HSP as vaccine candidates have been studied in other important diseases like *Plasmodium yoelii* (Sanchez et al. 2001), *Brugiamalayi* (Dakshinamoorthy et al. 2012), *Leishmania donovani* (Kaur et al. 2011) and Hantaan virus (Li et al. 2008).

A DNA vaccine based on the major capsid protein VP2 of infectious bursal disease virus (IBDV) induced both humoral and cell mediated immune responses, but with 40 to 80% protective efficacy that resulted in bursal lesions causing immunosuppression (Negash et al. 2013). However, fusing the c-terminal $HSP70_{359–610}$ gene genetically to VP2 gene not only resulted in enhanced antigen specific humoral and cell mediated immunity but also accorded complete protection of chickens against vvIBDV challenge as depicted in Fig. 4.1 (Maity et al. 2015). In another study, complete protection could be conferred against the lethal challenge with mouse-adapted H1N1, H3N2 or H9N2 influenza virus when the c-terminus of mHSP70 was fused with four tandem repeats of the ectodomain of the conserved influenza matrix protein M2 expressed in *Escherichia coli* and used as a vaccine candidate (Ebrahimi et al. 2012).

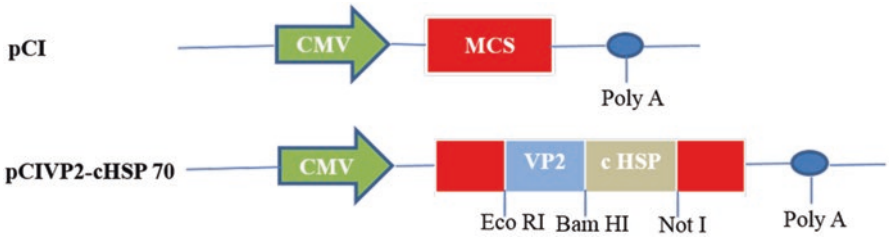

Fig. 4.1 Schematic representation of the fusion protein construct of VP2 gene of infectious bursal disease virus (IBDV) and c-terminal sequence of HSP70 of Mycobacterium tuberculosis. The fused gene fragment insertion in the eukaryotic expression vector pCI (Promega, USA) and expression in Vero cell line resulted in a cHSP70-VP2 antigenic complex of 72 kDa

The HSP70s of parasites including *Eimeria tenella*, *Toxoplasma gondii* and *Plasmodium falciparum* also act as adjuvants and enhanced the TLR2 and TLR4 responses resulting in Th1 type of response with activation of B cells and dendritic cells (Zhang et al. 2012; Mun et al. 2003; Qazi et al. 2005). Immunization of chickens with *E. tenella* antigen microneme protein 2 and EtHSP70 reduced the faecal oocyst shedding, enhanced body weight gain and elicited higher antibody responses against avian coccidiosis.

In another study, 65–70% protection could be afforded against lethal doses of *Salmonella* Typhi and *Salmonella* Typhimurium when recombinant GroEL (rGroEL) or HSP60 was injected. The antibody titers were boosted up along with increased T-cell proliferative responses and secretion of both Th1 and Th2 cytokines when the rGroEL was co-administered with recombinant IL-22 (rIL22) resulting in an increased protection efficacy of upto 90%. The increased protective efficacy could be attributed to the fact that IL-22 being a member of the IL-10 family of cytokines activated the Th17, Th1 and Th2 cells, CD4+ T cells, natural killer cells and natural killer T cells (Wolk and Sabat 2006). Several studies have indicated the importance of IL-22 in infections of the lung and intestine including *Klebsiella pneumonia* (Aujla et al. 2008), *Citrobacter rodentium* (Zheng et al. 2008), *Salmonella Typhimurium* (Schulz et al. 2008) and *Mycobacterium tuberculosis* (Dhiman et al. 2009).

An intranasal vaccination with a fused nucleoprotein (NP), matrix protein (M1) and HSP 60 (NP-M1-HSP60) construct expressed from *E. coli* in female BALB/c mice along with oil-in-water SP01 adjuvant resulted in humoral, mucosal, and cell-mediated immune responses. The vaccine candidate elicited high titers of specific IgG antibodies and Th1/Th2-associated immune responses and provided complete protection against lethal H7N9 virus challenge as there was significant decrease in viral replication, increase in survival rates and increased alleviation of lung pathology in challenged mice (Yang et al. 2014).

Heat shock protein gp96 (glucose-regulated protein, gp96) is present abundantly in the endoplasmic reticulum. The HSP could bind the antigenic peptides that are derived from tumors, viruses, intracellular bacteria and also helps in

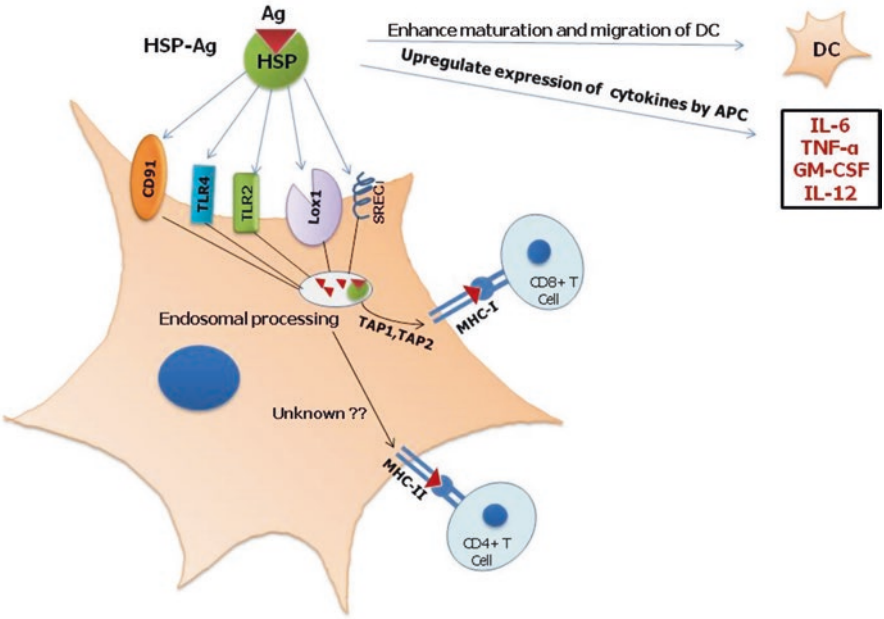

Fig. 4.2 Schematic diagram of antigen presentation pathway by HSP-Ag complex: HSP–Ag complex bind with the surface receptor CD91, SREC I, Lox1 either individually or together and might be associated with the TLR4 and TLR2 followed by internalization into the DCs. Following internalisation in early endosome the HSP-Ag complex might be processed and presented with MHC-I through Transporter associated with antigen processing (TAP) proteins, TAP1 and TAP2 to CD8+ T cells. Alternatively, processed antigenic fragment could also be loaded with MHC-II molecule as classical antigen presentation pathway and present to CD4+ T cells. HSP-Ag complex also enhance the maturation and migration of dendritic cells towards draining lymph nodes and upregulate the expression of cytokines like IL-6, TNF-α, GM-CSF, IL-12 by antigen presenting cells

cross-presentation of the peptides to MHC class I molecules as depicted in Fig. 4.2. This leads to activation of peptide-specific cytotoxic T lymphocyte mediated immune responses (Basu et al. 2001; Berwin et al. 2003; Binder and Srivastava 2004; de Filippo et al. 2008; Matsutake et al. 2010). It can also interact with TLR2, TLR4 and TLR9, thus inducing the secretion of TNF, IL-1 and IL-12 from APCs, resulting in activation of the innate immunity (Warger et al. 2006; Yang et al. 2007; Wu et al. 2012).

Immunization of heat shock protein gp96 with a pandemic H1N1 split vaccine led to a dramatic increase in antigen-specific T cell responses. The inactivated and split influenza virus vaccines alone mainly induced Th2 responses with minimal levels of Th1 responses but when co-administered with gp96 as an adjuvant significantly enhanced the Th1 immune responses as evidenced by higher IgG2aAb titers, higher numbers of IFN-γ CD8+ T cells, and a dramatic increase in viral-specific T cell responses in ELISPOT assays. Further, the cellular immunity elicited by the addition of gp96 to split H1N1 vaccine could cross-protect BALB/c mice against

the heterologous virus challenge. The effective rate of protection against infection with a lethal dose of a different subtype reached 100% after immunization with the gp96-adjuvanted vaccine (Ju et al. 2014).

The HSP70 plays an important role in the regulation of the life cycle of influenza virus. It binds to the viral ribonucleoprotein (vRNP) complex and prevents M1 (matrix protein) from binding to vRNP complex. Hence, the nuclear export of vRNP is inhibited (Hirayama et al. 2004). By interfering with the integrity of RNP, the HSP negatively regulate the viral transcription and replication (Li et al. 2011). It also interferes with the viral polymerase activity. Due to its vast importance in the regulation of the life cycle of influenza virus, the HSP could be developed as a potential anti-influenza drug candidate.

4.9 Role of Heat Shock Proteins in Immune-Surveillance and Cancer Immunotherapy

Cross-presentation of HSP chaperoned peptides by APCs occur efficiently to prime the cytotoxic T cell responses even with low doses of tumor antigens. Endocytic receptor CD91 plays a major role in the antigen transfer and HSP-peptide-CD91 mode of antigen transfer is proposed for the cross-presentation (Binder 2014). Upon stimulation, APCs secrete cytokines such as IL-1β, TNF-β, IL-6, IL-12 and GM-CSF and also up-regulate CD80, CD86, CD40 and MHC-II. The release of multiple HSP and presence of APCs results in Th1 type of response in the tumor microenvironment, which is capable of rejecting the tumor (Binder 2014).

Immunization with mycobacterial HSP70 gene fused with a fragment of the ovalbumin gene led to CTL response and tumor rejection (Suzue et al. 1997). In another study, the HSP from tumor cells and not the normal cells could elicit an immune response when the tumor specific peptides were fused to gp96 (Parmiani et al. 2004; Chen et al. 2004). Udano and Srivastava in 1993 showed that mice immunized with HSP70 purified from a tumor were protected from subsequent tumor challenge. Thus, HSP can not only be used as molecular adjuvants against a number of pathogens, they also play a pivotal role in tumor immunology and are helpful in combating various tumors.

HSP-peptide complexes can be good tumor vaccine candidates because of their capacity to bind to many tumor antigens, targeted delivery to APCs, cross-presentation of the delivered peptides, direct activation of APCs particularly DCs, generation of CD8+ T cell anti-tumor responses without the help of CD4+ T cell and effective activation of innate immune elements (Lee et al. 2006). HSP, especially gp96 and HSP70, extracted from tumors potentially induced tumor-specific CTL responses in murine models. Large HSP bind to and target protein antigens for DC mediated cross-presentation and results in enhanced efficacy of cancer vaccines. HSP also play important roles in molecular mechanisms that can lead to cancer development and metastasis. Further, they have been used as potential biomarkers for cancer diagnosis as well as therapeutic targets. Inhibitors of HSP have been tried

as novel anti-cancer agents. Different HSP family members collaborate with each other and regulate cellular functions. Therefore, a combination of different HSP inhibitors can enhance anti-cancer efficacy (Wu et al. 2017).

4.10 Conclusions

Veterinary vaccinology research is constantly evolving and is yet to discover its fullest potential. The cost of investment into vaccine research against important diseases of livestock including poultry either by public or private sector is minimal compared to its human counterparts. The quest for developing a vaccine with limited funding is a challenge for the scientific community involved in animal research across the globe. In this scenario, discovery of immunopotentiating agents in the form of adjuvants gain prominence. HSP molecules with a myriad of physiological functions aptly fit themselves as potential adjuvant candidates and have proven successful in immunopotentiating some of the veterinary vaccine candidates in experimental studies as enumerated in this review. The process of refining these molecules is the subject of recent research as evidenced by the number of publications in scientific research. It is a matter of time that these HSP molecules enter the main stream veterinary vaccine market and replaces the existing commercial adjuvants.

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Part II
Poultry Animals

Chapter 5
Antioxidant Systems and Vitagenes in Poultry Biology: Heat Shock Proteins

Peter F. Surai and Ivan I. Kochish

Abstract Commercial poultry production is associated with various stresses decreasing productive and reproductive performance of layers. A growing body of evidence indicates that most of stresses in poultry production at the cellular level are associated with oxidative stress due to excess of free radical production or inadequate antioxidant protection. Recently, a concept of the cellular antioxidant defence has been revised with a special attention paid to cell signalling. Antioxidant systems of the living cell is based on three major levels of defence and include several options and vitagene activation in stress conditions is considered as a fundamental adaptive mechanism. The vitagene family includes various genes responsible for synthesis of protective molecules such as thioredoxins, SOD, sirtuins and heat shock proteins (HSP). Indeed, HSP70, HSP90 and HSP32 (heme oxygenase) are among important elements of the antioxidant system network. However, by the time of writing no comprehensive review on the roles and effects of HSP in poultry biology has appeared. Therefore, the aim of this review is a critical analysis of the role of HSP in poultry biology with a specific emphasis to their functions as an essential

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part of the vitagene network. From the analysis of the recent data related to HSP in poultry physiology and adaptation to stresses it is possible to conclude that: a) HSP as important vitagenes are main driving force in cell/body adaptation to various stress conditions. Indeed, in stress conditions synthesis of most cellular proteins decreases while HSP expression is usually significantly increased; b) HSP as cellular chaperones are responsible for proteostasis and involved in protein quality control in the cell to prevent misfolding or to facilitate degradation, making sure that proteins are in optimal structure for their biological activities; c) there are tissue-specific differences in HSP expression which also depends on the strength of such stress-factors as heat, heavy metals, mycotoxins and other toxicants; d) HSP70, HSP90 and HSP32 are shown to be protective in heat stress, toxicity stress as well as in other oxidative-stress related conditions in poultry production; e) molecular mechanisms of HSP participation in acquisition of thermotolerance need further detailed investigation; f) there are complex interactions inside the antioxidant network of the cell/body to ensure an effective maintenance of homeostasis in stress conditions. Indeed, in many cases nutritional antioxidants (vitamin E, ascorbic acid, selenium) in the feed can decrease oxidative stress and as a result HSP expression could be decreased as well; g) regulating effects of various phytochemicals on HSP need further investigation; h) protective effects of HSP in immunity in stress conditions await practical applications in poultry production; i) nutritional means of additional HSP upregulation in stress conditions of poultry production and physiological and commercial consequences await investigation; j) vitagene upregulation in stress conditions is emerging as an effective means for stress management.

Keywords Antioxidant system · Chicken · HSP · Poultry · Stress · Vitagenes

Abbreviations

NF-κB	Nuclear factor-kappa B
AO	Antioxidant
ARE	Antioxidant response element
CAT	Catalase
ED	Embryonic day
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GST	Glutathione transferase
HO	Heme oxygenase
HS	Heat stress
HSF	Heat shock factor
HSP	Heat shock protein
HSR	Heat shock response
LDH	Lactate dehydrogenase
MDA	Malondialdehyde
NOS	Nitric oxide synthase
Nrf2	Nuclear factor-erythroid-2-related factor 2

PAO	Phenylarsine oxide
PPARα	Peroxisome proliferator activated receptor alpha
SOD	Superoxide dismutase
TM	Thermal manipulation (TM)

5.1 Introduction

Commercial poultry production is associated with various stresses decreasing productive and reproductive performance of layers. A growing body of evidence indicates that most of stresses in poultry production at the cellular level are associated with oxidative stress due to excess of free radical production or inadequate antioxidant protection. Recently, a concept of the cellular antioxidant defence has been revised with a special attention paid to cell signaling. Antioxidant systems of the living cell is based on three major levels of defense and include several options (Surai 2015a): Decrease localized oxygen concentration; decrease activity of prooxidant enzymes and improve efficiency of electron chain in the mitochondria and decreasing electron leakage leading to superoxide production; prevention of chain initiation by scavenging initial radicals due to inducing various transcription factors (e.g., Nrf2, NF-κB and others) and ARE-related synthesis of AO enzymes (SOD, GSH-Px, CAT, glutathione reductase (GR), GST, etc.); binding metal ions (metal-binding proteins) and metal chelating; decomposition of peroxides by converting them to non-radical, nontoxic products (Se-GSH-Px); chain breaking by scavenging intermediate radicals such as peroxyl and alkoxyl radicals (vitamins E, C, GSH, uric acid, ubiquinol, bilirubin, etc.); repair and removal of damaged molecules (methionine sulfoxide reductase, DNA-repair enzymes, chaperons, etc.) and vitagene activation and synthesis and increased expression of protective molecules (GSH, Thioredoxins, SOD, heat shock proteins (HSP), sirtuins, etc.).

Indeed, understanding roles of vitagenes in stress resistance of poultry as a background for the development of effective strategies to deal with stresses is an emerging topic of research (Surai 2015a, b, c; 2014; Shatskikh et al. 2015; Surai and Fisinin 2015). It is known that vitagenes are responsible for synthesis of various protective molecules and HSP synthesis is under vitagene control. However, by the time of writing no comprehensive review on the roles and effects of HSP in poultry biology has appeared. Therefore, the aim of this review is a critical analysis of the role of HSP in poultry biology with special emphasis to their functions as an essential part of the vitagene network, responsible for adaptive ability of the cells or whole organisms to various stress conditions.

5.1.1 Heat Shock Response and Heat Sock Factors

The heat shock response (HSR) is one of the main adaptive stress responses of the cell, restoring cellular homeostasis upon exposure to proteotoxic stress, including heat shock, cold, oxidative stress, hypoxia, toxins, chemicals, pathogen, etc.

Table 5.1 Chemical and physical inducers with their target genes in the stress response pathway (Adapted from Surai 2002, 2014, 2015a, c; Gupta et al. 2010)

Stress response pathway	Target genes	Chemical and physical inducers
Heat shock response	*Hsp70*, *Hsp90*, *Hsp60*, *Hsp26*, HSP32, *Hsp27*, *Hsp23*	High temperature, pesticides, heavy metals, solvents, industrial and municipal effluents, mono and polycyclic aromatic hydrocarbons, mycotoxins
Oxidative stress	*SOD*, *Catalase*, *Thioredoxin and TR*, *Peroxiredoxin, GSH and GSH-Px, Nrf2*	Heavy metals, quinines, and volatile organic solvents, mycotoxins, peroxides, phytochemicals, high temperature, Fe overload
DNA damage response	*p53*, *RecA/Rad51*, *MutL/MLH*, *MutS/MSH*	Pesticides, methyl methanesulfonate, and cyclophosphamide
Metal stress	*Metalothionine*	Heavy metals
Inflammation	*NF-κB*, *STAT3*, *AP-1*, *TNF-α*, *IL-1β*, *IL-6*	Metals, polychlorinated biphenyls (PCBs), exhaust particles, and smoke particles
Hypoxia	*P38*, *pI3k*	Metals and quercetin
Xenobiotics metabolism	*Cyps*, *GSH system*	Pesticides, heavy metals, volatile organic solvents, alkaloids, caffeine, and phenobarbital

Table 5.1 (Pockley and Multhoff 2008; Velichko et al. 2013; Meijering et al. 2015). In fact, cooperative interactions between the transcription factors and various homeostatic mechanisms are responsible for effective adaptation to stressful conditions (Fujimoto and Nakai 2010; Sakurai and Enoki 2010; Takii et al. 2015). Indeed, to maintain vital life function it is imperative that organisms preserve the integrity of their proteins. Therefore, HSR in vertebrates is characterized by the induction of HSP and related elements, such as the ubiquitin–proteasome system (Velichko et al. 2013). Because HSP act as molecular chaperones that facilitate protein folding and suppress protein aggregation, this response plays a major role in maintaining protein homeostasis. Generally, HSR is regulated mainly at the level of transcription by four heat shock transcription factors (HSFs), including HSF1, HSF2, HSF3, and HSF4, which bind to HSE (Fujimoto and Nakai 2010), thus resulting in stimulation of HSP expression.

Among other heat shock factors, HSF1 has received tremendous attention as the main factor governing the HSR by coordinating stress-induced transcription (Richter et al. 2010). Although originally discovered as a response to thermal stress, HSR can be triggered by a variety of stress conditions that interfere with protein folding and result in accumulation of misfolded or aggregated proteins (Liu and Chang 2008). HSF1 activation is a multistep process that is negatively regulated by chaperones, including HSP90 and HSP70 (de Thonel et al. 2012), which sets the stage for rapid induction of gene expression within minutes of cellular stress (Stetler et al. 2010). In physiological conditions the majority of HSFs form a complex with HSP70 or HSP90 interacting with the HSF1 activation domain. In stress conditions, HSP70 and HSP90 form complexes with denatured proteins, which releases HSFs (Kantidze et al. 2015). Furthermore, in unstressed state, HSF1 is present in the cytoplasm as a latent monomeric molecule. Upon heat shock, monomeric HSF1 is

hyperphosphorylated and converts to a trimer with the capacity to bind DNA that accumulates in the nucleus and subsequently binds to the heat shock element within the promoter region of HSP genes. In addition, extensive posttranslational modifications such as phosphorylation, acetylation, and sumoylation are thought to fine-tune HSF1 activity (Meijering et al. 2015; Takii et al. 2015). The increased expression of HSP continues until the amount of HSP70 and HSP90 reaches the level sufficient to block the activation domain of the HSFs (Kantidze et al. 2015).

5.1.1.1 Chicken HSF

Avian cells express at least three HSFs (HSFs 1–3). Initially, three avian HSF genes corresponding to a novel factor, HSF3, and the avian homologs of mammalian HSF1 and HSF2 have been cloned (Nakai and Morimoto 1993). The predicted amino acid sequence of HSF3 is approximately 40% related to the sequence of HSF1 and HSF2. Similar to HSF1 and HSF2, the HSF3 message, is coexpressed during development and in most tissues, which suggests a general role for the regulatory pathway involving HSF3 (Nakai and Morimoto 1993). It was shown that the regulatory domain is located between the transcriptional activation domains and the DNA binding domain of HSF1 and is conserved between mammalian and chicken HSF1 but is not found in HSF2 or HSF3 (Green et al. 1995). Indeed, the regulatory domain was found to be functionally homologous between chicken and human HSF1. In fact, HSF3 is negatively regulated in avian cells and acquires DNA-binding activity in certain cells upon heat shock (Nakai et al. 1995). Induction of HSF3 DNA-binding activity is delayed compared with that of HSF1 and heat shock leads to the translocation of HSF3 to the nucleus (Nakai et al. 1995). It has been shown that HSF1 is rapidly activated by even mild heat shock, while HSF3 is activated only by severe heat shock. In contrast, HSF2 is not activated by heat stress and has been speculated to have developmental functions (Tanabe et al. 1997).

Indeed, cHSF3 (chick HSF3) was activated at higher temperatures than the cHSF1. In fact, at a mild heat shock, such as 41 °C, only cHSF1 was activated, whereas both cHSF1 and cHSF3 were activated following a severe heat shock at 45 °C. Similarly, cHSF3 was activated by treating cells with higher concentrations of sodium arsenite compared to cHSF1. Furthermore, the DNA binding activity of cHSF3 by severe heat shock lasted for a longer period than that of cHSF1. In addition, the total amount of cHSF3 increased only upon severe heat shock, whereas that of HSF1 decreased. Indeed, cHSF3 is involved in the persistent and burst activation of stress genes upon severe stress in chicken cells (Tanabe et al. 1997). It seems likely that denaturation of nascent polypeptides could be the first trigger for the activation of cHSF1 and cHSF3 (Tanabe et al. 1997). It has been suggested that HSF3 has a dominant role in the regulation of the heat shock response and directly influences HSF1 activity. Thus, disruption of the HSF3 gene results in the severe reduction of heat shock gene expression and loss of thermotolerance (Tanabe et al. 1998). In addition, null cells lacking HSF3, yet expressing normal levels of HSF1,

exhibited a severe reduction in the heat shock response, as measured by inducible expression of heat shock genes, and did not exhibit thermotolerance.

Important information related to HSFs in avian species has been obtained in experiments with chick embryos. In fact, it was shown that HSF3 was almost constantly expressed in various tissues during early to late chicken embryonic development (Kawazoe et al. 1999). The expression of HSF1 was equally high in most tissues early in development and thereafter declined to different levels in a tissue-dependent manner and HSF3 became the dominant heat-responsive factor mediating stress signals to heat shock gene expression in the chicken. Furthermore, the high-level and ubiquitous expression of HSF2 as well as HSF1 and HSF3 in early embryogenesis suggest the involvement of these factors in all developmental processes (Kawazoe et al. 1999). It is interesting to note that in avian, HSF1 and HSF3 are maintained in a cryptic monomer and dimer form, respectively, in the cytoplasm in the absence of stress. Upon heat stress, they undergo conformational change associated with the formation of a trimer and nuclear translocation and the nuclear localization signal acts positively on the trimer formation of cHSF3 upon stress conditions (Nakai and Ishikawa 2000). Indeed, avian cells express two redundant heat-shock responsive factors, HSF1 and HSF3, which differ in their activation kinetics and threshold induction temperature. For example, in birds, HSF1 only slightly induces HSP70 expression during heat shock and indeed HSF3 is a master regulator of the heat shock genes in avian cells, as is HSF1 in mammalian cells (Inouye et al. 2003). Avian cells lacking two heat-inducible HSFs, HSF1 and HSF3 were generated (Nakai and Ishikawa 2001). In addition to complete loss of activation of heat shock genes under stress conditions, these cells exhibited a marked reduction in HSP90α expression under normal growth conditions. Reduction in HSP90α expression caused instability of a cyclin-dependent kinase, Cdc2, and cell cycle progression was blocked mainly at the G2 phase, but also at G1 phase even at mild heat shock temperatures. Restoration of HSP90α expression rescued the temperature sensitivity without induction of HSP (Nakai and Ishikawa 2001). Whereas HSF1 mediates transcriptional activity only in the brain upon severe heat shock, HSF3 is exclusively activated in blood cells upon light, moderate, and severe heat shock, promoting induction of heat-shock genes (Shabtay and Arad 2006). Although not activated, HSF1 is expressed in blood cell nuclei in a granular appearance, suggesting regulation of genes other than heat-shock genes. It was shown that HSF1 and HSF3 mediate transcriptional activity of adult tissues and differentiated cells in a nonredundant manner. Instead, an exclusive, tissue-specific activation is observed, implying that redundancy may be developmentally related (Shabtay and Arad 2006). The heat shock response regulated by the HSF family should consist of the induction of classical as well as of nonclassical heat shock genes, both of which might be required to maintain protein homeostasis (Fujimoto and Nakai 2010). Recently, additional information on the roles of HSF2 has been obtained. In particular it has been shown that vertebrate HSF2 is activated during heat shock in the physiological range (Shinkawa et al. 2011). HSF2 deficiency reduces threshold for chicken HSF3 or mouse HSF1 activation, resulting in increased HSP expression during mild heat shock. HSF2-null cells are more sensitive to sustained mild heat

shock than wild-type cells, associated with the accumulation of ubiquitylated misfolded proteins. Furthermore, loss of HSF2 function increases the accumulation of aggregated polyglutamine protein and shortens the lifespan of R6/2 Huntington's disease mice, partly through αB-crystallin expression (Shinkawa et al. 2011). In fact, HSF2 was identified as a major regulator of proteostasis capacity against febrile-range thermal stress (Shinkawa et al. 2011). It was also shown that chicken HSF3, but not chicken HSF1, also induces the expression of the major avian pyrogenic cytokine IL-6 during heat shock (Prakasam et al. 2013)

In general, important roles of HSFs in adaptation of poultry to various stress conditions are difficult to overestimate. However, recent genome-wide studies have revealed that HSF1 is capable of reprogramming transcription more extensively than previously assumed; it is also involved in a multitude of processes in stressed and non-stressed cells (Vihervaara and Sistonen 2014).

5.1.2 Heat Shock Proteins

Heat shock proteins (HSP) are highly conserved families of proteins discovered in 1962 (Ritossa 1962). Later, it has been realized that most HSP have strong cytoprotective effects and are molecular chaperones for other cellular proteins. Taking into account current knowledge of the mode of action of HSP, the name of "stress proteins" would be more appropriate for them but due to historical reasons they are still called HSP. Indeed, in the case of oxidative stress, HSP network participates in detecting intracellular changes, protecting against protein misfolding and preventing activation of downstream events related to inflammation and apoptosis (Fig. 5.1; Kalmar and Greensmith 2009). Since oxidative stress plays a major role in a number of diseases and disease mechanisms in human (Kalmar and Greensmith 2009) and decreases productive and reproductive performance in farm animals (Surai 2006), it is likely that any medication/treatment that is able to reduce levels of oxidative stress will make a significant impact on human health and animal performance. Some HSP are constitutively expressed, whereas others are strictly stress-inducible. Under physiologic conditions, HSP play an important role as molecular chaperones by promoting the correct protein folding and participating in the transportation of proteins across intracellular membranes and repair of denatured proteins. Therefore, HSP participate in the regulation of essential cell functions, such as protein translocation, refolding, assembly and the recognition, prevention of protein aggregation, renaturation of misfolded proteins, degradation of unstable proteins, etc. (Zilaee et al. 2014). It should be mentioned that the events of cell stress and cell death are linked and HSP induced in response to stress appear to function at key regulatory points in the control of apoptosis (Garrido et al. 2001; Kennedy et al. 2014). A key feature of HSP is their ability to provide cytoprotection. Synthesis of these proteins under stress conditions is a highly conserved mechanism of the cell response and adaptation is common among all living organisms. In fact, HSP are synthesized in response to a great variety of cellular stresses, including heat stress, hypoxia,

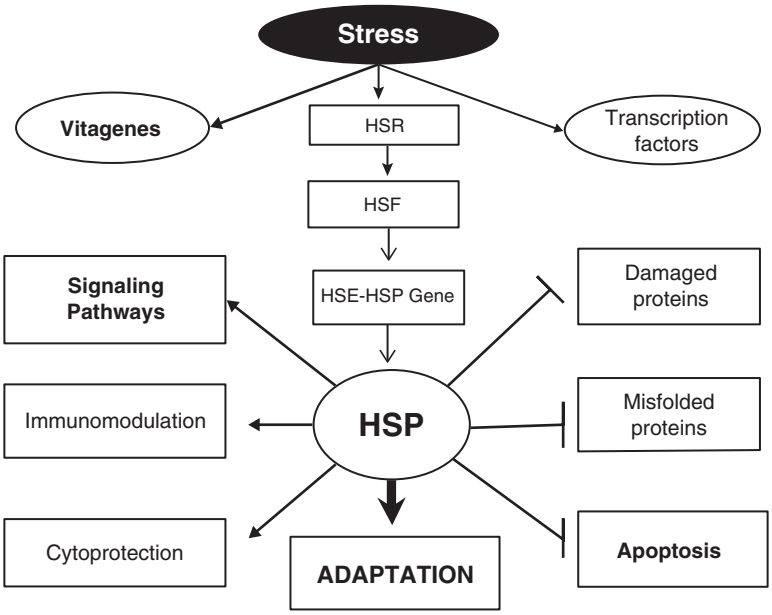

Fig. 5.1 Functions of HSP under stress conditions (Adapted from Surai 2015c and Khalil et al. 2011)

ischemia, hypothermia, virus infections as well as the effects of various toxicants, including mycotoxins (Velichko et al. 2013). It is important to note that upregulation of the synthesis of HSP is considered an endogenous adaptive phenomenon leading to improved tolerance to various stress conditions/factors. In mammals and birds the HSP superfamily includes five broadly conserved families of proteins Table 5.2. Among them HSP70, HSP90 and HSP32 (HO-1) are considered as vitagenes.

5.1.2.1 Heat Shock Protein 70 (HSP70)

Among the HSP, HSP70 is one of the most conserved and important protein families and has been extensively reviewed (Mayer 2013; Shiber and Ravid 2014; Duncan et al. 2015; Qu et al. 2015) and will be briefly dealt with here. HSP70 refers to a family of 70 kDa chaperone proteins. Some of the important house-keeping functions attributed to HSP70 include (Garrido et al. 2006): import of proteins into cellular compartments; folding of proteins in the cytosol, endoplasmic reticulum and mitochondria; degradation of unstable proteins; dissolution of protein complexes; control of regulatory proteins; refolding of misfolded proteins and translocation of precursor proteins into mitochondria. These molecular chaperones are implicated in a wide variety of cellular processes, including protein biogenesis, protection of the proteome from negative consequences of stress, recovery of

Table 5.2 . Mammalian heat shock proteins (Adapted from Bozaykut et al. 2014 and O'Neill et al. 2014)

HSP family	Location	Summary of structural features and domains	Main established functions
HSP90	cyt	Homodimer with two cytosolic isoforms α and β, dimerization occurs at the C-terminal and nucleotide exchange at the N-terminal	Chaperone for a multitude of client proteins and regulator of protein complex formation; mainly responsible for cell viability, keeps proteins in folded state
HSP70	cyt/ nucl/ mit	Consists of a N-terminal (ATPase domain) and a C-terminal substrate-binding domain connected by a short flexible linker	Protein trafficking and degradation, refolding of denatured proteins; during stress anti-apoptotic properties; protein quality control and turnover
HSP60	mit	Arranged as two stacked heptameric rings with three domains (apical, intermediate and equatorial)	Mitochondrial protein folding and assembly
HSP40	cyt/ nucl	J-domain that stimulates the ATPase activity of Hsp70 and C-terminal that loads polypeptides to Hsp70	Regulates activity of Hsp70; binds non-native proteins; processes pro-collagen; substrate delivery to HSP70, targets nonnative proteins to ERAD
sHSP	cyt	Conserved C-terminal and highly variable N-terminal (WDPF domain)	Preventing unfolded protein aggregation; prevent the accumulation of aggregated proteins

cyt cytosol, *nucl* nuclear, *mit* mitochondria

proteins from aggregates, facilitation of protein translocation across membranes, as well as disassembly of particular protein complexes and cell signaling for growth, differentiation, and apoptosis (Clerico et al. 2015). In particular, HSP70 can inhibit apoptosis by interfering with target proteins (Ravagnan et al. 2001). In eukaryotic cells, HSP70s are subject to a large number of post-translational modifications (Mayer 2013). These ATP-dependent chaperones represent central components of the cellular protein surveillance network and are involved in a large variety of protein-folding processes. In fact, they effectively interact with practically all proteins in their unfolded, misfolded, or aggregated states but do not interact with their folded counterparts (Mayer 2013). A number of eukaryotic proteins are regulated through transient association with HSP70, including steroid hormone receptors, kinases and transcription factors. Eight different and unique HSP70 have been identified in eukaryote cells being distributed in different subcellular compartments, including cytosol, nucleus, endoplasmic reticulum, and mitochondria (Daugaard et al. 2007; Mahalka et al. 2014). The two most important members of the HSP70 family are the constitutively expressed 73 kDa heat shock cognate (HSC73, HSC70, HSPA8) and stress-inducible 72 kDa heat shock protein (HSP72, HSP1A) (Meimaridou et al. 2009). Indeed, under normal conditions HSP70 proteins function as ATP-dependent molecular chaperones maintaining important cell functions related to proteostasis (Mayer 2013). Under various stress conditions additional synthesis of stress-inducible HSP70 enhances the ability of stressed cells to deal

with increased concentrations of unfolded or denatured proteins (Clerico et al. 2015). HSP70 expression is associated with a reduction in JNK1 phosphorylation and/or an increase in oxidative capacity consequential of improvements in mitochondrial homeostasis (Henstridge et al. 2014). It seems likely that HSP70s do not work alone but with a team of cochaperones. Recently it has been found that the organelle distribution of HSP70 is determined by their specific lipid compositions. In particular, HSP70 attach to lipids by extended phospholipid anchorage, with specific acidic phospholipids associating with HSP70 in the extended conformation with acyl chains inserting into hydrophobic crevices within HSP70, and other chains remaining in the bilayer (Mahalka et al. 2014). It seems likely that this could represent an important connection between HSP and lipid quality control in the cell and the HSP90/HSP70-based chaperone machinery may function as a comprehensive protein management system for quality control of damaged proteins. Actually in a recently developed model, it was proposed that the heat shock protein HSP90/HSP70-based chaperone machinery played a major role in determining the selection of proteins that have undergone oxidative or other toxic damage for ubiquitination and proteasomal degradation (Pratt et al. 2010).

5.1.2.1.1 Chicken HSP70

In 1978 it was shown that the pattern of proteins synthesized by chicken embryo fibroblasts changes dramatically after heat treatment (45 °C for a few hours). In fact, three proteins (Mr = 22,000, 76,000, and 95,000) accounted for almost 50% of the cell's protein synthetic capacity immediately after the heat-shock (Kelley and Schlesinger 1978). The university of the heat shock response and conservation of proteins induced by this type of stress was proven in different experimental conditions. In particular, antibodies to chicken HSP, cHSP89 and cHSP70, cross-reacted with proteins of similar molecular weights in embryonic and adult chicken tissues and in extracts from widely different organisms ranging from yeast to mammals (Kelley and Schlesinger 1982). Heat-shock polypeptides of identical sizes of 85,000, 70,000, and 25,000 Da were synthesized predominantly in chicken embryo fibroblasts and in many different organs of 18-day-old embryos at 42.5–44 °C (Voellmy and Bromley 1982). Effects of heat treatments on chick embryo fibroblasts, Drosophila embryonic cells, and human lymphoblastoid cells have been compared (Voellmy et al. 1983). Cells from all three species synthesize large HSP with Mr = 70,000 and 84,000–85,000. Different small HSP with Mr between 22,000 and 27,000 are made at high rates in heat-treated chicken and Drosophila cells but could not be observed in human cells. It was found that chicken reticulocytes respond to elevated temperatures by the induction of only one heat shock protein, HSP70, whereas lymphocytes induce the synthesis of all four heat shock proteins (HSP89, HSP70, HSP23 and HSP22). The synthesis of HSP70 in lymphocytes was rapidly induced by small increases in temperature (2–3 °C) and blocked by preincubation with actinomycin D (Morimoto and Fodor 1984). Furthermore, incubation of chicken reticulocytes at elevated temperatures (43–45 °C) resulted in a rapid change

in the pattern of protein synthesis, characterized by the decreased synthesis of normal proteins, e.g., alpha and beta globin, and the preferential and increased synthesis of HSP70 (Banerji et al. 1984). Indeed, the rapid 20-fold increase in the synthesis of HSP70 was observed after heat shock and preincubation of reticulocytes with the transcription inhibitor actinomycin D or 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole blocked the heat shock-induced synthesis of HSP70.

In 1986 Morimoto and co-authors studied organization, nucleotide sequence, and transcription of the chicken HSP70 gene. They isolated a gene encoding a 70,000-Da heat shock protein (HSP70) from a chicken genomic library and showed that the order and spacing of the sequences share many features in common with the promoter for the human HSP70 gene (Morimoto et al. 1986). The expression of HSP70 during maturation of avian erythroid cells was also studied (Banerji et al. 1987). It was shown that definitive red cells respond to heat shock by a 10- to 20-fold increase in HSP70 protein synthesis with little change in HSP70 mRNA levels. Therefore, the increased expression of HSP70 in cells was due to increased translatability of HSP70 mRNA. Furthermore, the authors showed that HSP70 expression in erythroid cells is lineage specific and although HSP70 was constitutively expressed, neither HSP70 synthesis nor HSP70 mRNA levels were heat shock inducible in primitive red cells.

HSP70 was shown to constitutively express in the embryonic chicken lens. In fact, HSP70 mRNA in the embryonic chicken lens was associated primarily with cells in the early stages of fiber formation, and increased transcription of this gene was part of the differentiation process (Dash et al. 1994). It was shown that the heat induced increase in HSP70 mRNA and protein in broiler liver, in vivo, are time dependent, similar to that in mammals (Gabriel et al. 1996). An increase in the amount of HSP70 was detected from the first up to the fifth hour of acute heat exposure (35 °C for 5 h), while an increase in HSP70 mRNA peaked at 3 h. It seems likely that heat shock response in avian species is related to temperatures above 41 °C. For example, the spatial expression of HSP70 transcripts was detected in chicken embryos under normal incubation conditions and moderate heat stress (41 °C) did not induce enhancements on HSP70 mRNA levels (Gabriel et al. 2002). At the same time, acute exposure to severe heat stress (44 °C) for one hour resulted in a fifteen-fold increase in HSP70 mRNA levels. It is interesting to note that the return of stressed embryos to normal incubation temperature resulted in increased HSP70 mRNA levels for three hours which was normalised after six hours. The increased expression of HSP70 in broiler chicken embryos was shown to be affected not only by heat (41 °C) but also by cold (32 °C) stress, and is tissue- and age-dependent (Leandro et al. 2004). In fact, HSP70 was detected in the liver, heart, breast muscle, and lungs and the brain contained 2- to 5-times more HSP70 protein compared to the other embryonic tissues. These data are in agreement with our observations indicating low level of vitamin E and high levels of PUFAs in chicken embryonic brain (Surai et al. 1996). Therefore, increased HSP70 expression is an adaptive mechanism of increasing antioxidant defences. Younger embryos had higher HSP70 synthesis than older embryos, irrespective of the type of thermal stressor (Leandro et al. 2004). Again, these data confirm our finding about matura-

tion of the antioxidant defences during chicken embryonic development (Surai 1999a).

It was shown that HSP70 expression in postnatal chickens is tissue- and allele-dependent (Zhen et al. 2006). Indeed, the expression of HSP70 gene in the liver was significantly (more than 2-fold) higher than that in the muscle under normal growth conditions. This could reflect an importance of HSP70 chaperone functions, since the liver is the major site of synthesis of many important proteins. However, during acute heat stress (44 °C for 4 hours) the expression of HSP70 gene in the brain was the highest being significantly different from those in the liver and muscle. This adaptive response by HSP70 also is an important mechanism to compensate for relatively low levels of antioxidants in the brain tissue of the chicken (Surai 2002). Long-term, moderate heat stress (30–32 °C) was associated with significantly increased HSP70 levels in mononuclear blood cells of laying hens (Maak et al. 2003). However, the age-dependent responses of different genotypes were not uniform. HSP70 gene expression was gender-dependent with significantly higher levels in male than in female chickens (Figueiredo et al. 2007) and tissue-dependent heat induction of HSP70 expression may correlate with DNA methylation pattern in the HSP70 promoter (Gan et al. 2013b). During the exposure to heat stress (37±1 °C), the heart, liver and kidney of broiler chickens exhibited increased amounts of HSP70 protein and mRNA. The expression of HSP70 mRNA in the heart, liver and kidney of heat-stressed broilers increased significantly and attained the highest level after a 2-h exposure to elevated temperatures. Significant elevations in HSP70 protein occurred after 2, 5, and 3 h of heat stressing, respectively, indicating that the stress-induced responses vary among different tissues (Yu et al. 2008). Furthermore, the expression of HSF3 and HSP70 mRNA in Lingshan chickens (LSC) and White Recessive Rock (WRR) exhibited species-specific and tissue-specific differences during heat treatment (Zhang et al. 2014). For example, after 2 h of heat treatment, HSP70 expression was significantly higher in the liver and leg muscle of WRR compared to LSC. Recent analysis of genetic diversity of the HSP70 gene in 8 native Chinese chicken breeds indicates presence of 36 variations, which included 34 single nucleotide polymorphisms and 2 indel mutations (Gan et al. 2015). Furthermore, 57 haplotypes were observed, of which, 43 were breed-specific and 14 were shared.

HSP expression in the gut could be considered as an important mechanism of the antioxidant protection (Surai and Fisinin 2015). However, there were no effects of HSP70 overexpression on intestinal morphology under heat stress, but there was a strong correlation between HSP70 expression and the digestive enzyme activity in broilers (Hao et al. 2012). In another study from the same department, HSP70 induction was shown to protect the intestinal mucosa from heat-stress injury by improving antioxidant capacity of broilers and inhibiting the lipid peroxidation production (Gu et al. 2012). In fact, HSP70 significantly protected the integrity of the intestinal mucosa from heat stress (36 ± 1 °C) by significantly elevating antioxidant enzyme activities (SOD, GSH-Px and total antioxidant capacity) and inhibiting lipid peroxidation to relieve intestinal mucosal oxidative injury.

To investigate the alterations introduced by domestication and selective breeding in heat stress response, two experiments were conducted using Red Jungle Fowl (RJF), village fowl (VF), and commercial broilers (CB). Birds of similar age (30 d old) or common body weight (930 ± 15 g) were exposed to 36 ± 1 °C for 3 h (Soleimani et al. 2011). The RJF at a common age and common BW showed significantly higher levels of basal HSP70 and cortisone compared with VF and CB. Heat treatment was shown to significantly increase body temperature, heterophil:lymphocyte ratio, and plasma corticosterone concentration in CB but not in VF and RJF. Irrespective of stage of heat treatment, RJF showed lower heterophil:lymphocyte ratio and higher plasma corticosterone concentration than VF and CB. It was concluded that domestication and selective breeding are leading to individuals that are more susceptible to stress rather than resistant (Soleimani et al. 2011). Furthermore, laying hens exposed to HS (32.6 °C) showed higher concentrations of HSP70 in the liver (Felver-Gant et al. 2012). In addition, kind gentle hens (a line of group-selected hens for high productivity and survivability) had higher concentrations of HSP70 than DeKalb XL hens (commercial line of individually selected hens for high egg production) regardless of treatment.

HSP70 is also shown to be expressed in other avian species. Notably, quail HSP70 showed 98% homology with HSP70 stress protein in Gallus gallus and 99% homology with Numida meleageris (Gaviol et al. 2008). Duck HSP70 gene was also identified and characterised (GenBank: EU678246) and shown to contain no introns (Xia et al. 2013). Fifteen variations were identified within the open reading frame. The expression of duck HSP70 gene was tissue-specific and the highest expression level was seen in pectoral muscle (Xia et al. 2013).

To sum up, the results from the aforementioned studies consistently demonstrate that increased HSP70 expression in chicken tissues is one of the most important protective responses to prevent or deal with, detrimental changes in protein structure and functions due to various stresses. However, there is a need for further research to understand molecular mechanisms of HSP70 regulation in avian species.

5.1.2.2 Heat Shock Protein 90 (HSP90)

HSP90, the major soluble protein of the cell, has recently received great attention and a range of reviews described its structure, functions and regulation (Erlejman et al. 2014; Karagöz and Rüdiger 2015; Mayer and Le Breton 2015; Khurana and Bhattacharyya 2015). In fact, in the cell, HSP90 is known to comprise 1–2% of total proteins under non-stress conditions and it is further upregulated under stress (Csermely et al. 1998). For example, heat shock (37–42 °C) have been reported to induce HSP90 levels by as much as twofold (Bagatell et al. 2000). Furthermore, fish naturally living in a hot spring with relatively high water temperature (34.4±0.6 °C) is characterised by increased levels of all the studied HSP (HSP70, HSP60, HSP90, HSC70 and GRP75) compared with fish living in normal river water temperature (Oksala et al. 2014).

HSP90 is expressed as a 90 kDa protein and its functional molecule is a homodimer (α/α or β/β) and each monomer consists of three domains. They are NH2-terminal nucleotide binding domain (a binding site for ATP/ADP), the middle domain (the binding site for nuclear localization signal and client proteins) and the C-terminal domain (the site of dimerization and co-chaperone binding) (Li et al. 2012a, b). The HSP90 family in mammalian cells consists of four major homologs including two cytoplasmic isoforms HSP90α (inducible form) and HSP90β (constitutive form) (Sreedhar et al. 2004), HSP90B located in endoplasmic reticulum and tumor necrosis factor receptor-associated protein (TRAP) found in mitochondria and the inner membrane space (Revathi and Prashanth 2015). It is interesting to note that HSP90α and HSP90β share 86% amino acid identity and are expressed in all nucleated cells.

HSP90 is a highly efficient, ATP-dependent molecular chaperone involved in the maturation and stabilisation of a wide-range of proteins in both physiological and stress conditions being an important hub in the protein network that maintains cellular homeostasis and function (Jackson 2013). HSP90 belongs to a family of proteins known as "chaperones," which are solely dedicated to helping other proteins (client proteins) correct folding, function and stability. Indeed, cellular stress causes protein denaturation, and they cannot function properly and must be repaired or eliminated with the help of chaperones (Garcia-Carbonero et al. 2013). HSP90 deals with more than 200 important clients which are involved in signal transduction, including many steroid hormone receptors, receptor tyrosine kinases, Src family members, serine-threonine kinases, cell cycle regulators, telomerase and many other proteins (Li et al. 2012b; Wayne et al. 2011; Zhang and Burrows 2004). It is difficult to overestimate chaperoning functions of HSP90 related to various nuclear proteins regulating DNA replication, DNA repair, DNA metabolism, RNA transcription and RNA processing (Li et al. 2012b) and the protective action of HSP90 is related to posttranslational modifications of soluble nuclear factors as well as histones (Erlejman et al. 2014).

It was suggested that HSP90 clients are associated with major physiological events including signal transduction, cell cycle progression, transcriptional regulation, natural and acquired immunity and intracellular movement of proteins (Li et al. 2012b; Taipale et al. 2010). In fact, HSP90 participates in many cellular processes including cell cycle control, cell survival, hormone and other signaling transduction pathways, often acting as hormone receptors and is considered to be key player in maintaining cellular homeostasis and adaptive response to stress (Jackson 2013). In many cases, HSP90-assocoated stress response is orchestrated via HSF1, which under stress conditions upregulates several hundred genes including HSP90. It is known that under physiological condition, as a client protein, HSF1 is kept in an inactive monomeric form through the transient interaction with HSP90 (Li et al. 2012b). During stress, HSF1 dissociates from HSP90, homotrimerizes, undergoes phosphorylation and translocates to the nucleus to perform its gene-expression regulatory functions (Li et al. 2012b). As a matter of fact, HSP90 is regulated transcriptionally through direct interactions with the transcription factor HSF (Trinklein et al. 2004). Generally, HSP90 is present in cells in equilibrium between a low

chaperoning activity 'latent state' in physiological conditions and an 'activated state', with increased chaperoning efficiency in stress conditions (Chiosis et al. 2004).

HSP90 usually works as a complex with other chaperones and over 20 co-chaperones (Hong et al. 2013) and increased expression of HSP90 have been shown to be associated with the tolerance of hypothermia, cell proliferation, and cell cycle control (Herring and Gawlik 2007). In fact, co-chaperones assist HSP90 in its conformational cycling, act as substrate recognition proteins and provide additional enzymatic activity (Barrott and Haystead 2013). It seems likely that under heat stress conditions, co-chaperones allow HSP90 to prevent aggregation of unfolded proteins (Richter et al. 2010). Indeed, HSP90 involves in the folding, stabilization, activation and assembly of its client proteins through the formation of complexes with co-chaperones such as HSP70, HSP40, Hop, Hip and p23 (Whitesell and Lindquist 2005). The molecular chaperones HSP90 and HSP70 form a multichaperone complex, in which both are connected by a third protein called Hop. Indeed, Hop (HSP70/HSP90 organizing protein) facilitates interaction between HSP90 and HSP70 helping substrate to be efficiently transferred from HSP70 to HSP90 (Daniel et al. 2008). It seems likely that the interplay between the two chaperone machineries affecting the trafficking and turnover of several hundred signaling proteins as well as removal of damaged and aberrant proteins via the ubiquitin-proteasome pathway is of great importance for cell viability and adaptability. HSP90 is shown to possess an ATPase activity, which is known to be essential to modulate the conformational dynamics of the protein. In fact, ATP hydrolysis is associated with the HSP90 dimer transitioning into its "open" conformation and releasing the client protein (Taipale et al. 2010). The system is regulated by post-translational modifications including phosphorylation, acetylation, nitrosylation and methylation and uses a range of co-chaperones mediating interactions with HSP90 client proteins (Li et al. 2012b; Jackson 2013). Therefore, HSP90 has been considered to be a key factor at the crossroads of genetics and epigenetics (Erlejman et al. 2014).

5.1.2.2.1 Chicken HSP90

A cDNA clone for the 90 kDa heat-shock protein was isolated by direct immunological screening of a chicken smooth muscle cDNA expression library (Catelli et al. 1985a). It was shown that HSP90 is increased in heat-shocked chick embryo fibroblasts (Catelli et al. 1985b). Furthermore, HSP90 from chicken liver has been purified and physically characterized (Iannotti et al. 1988). The protein was shown to be an elongated dimer with a molecular weight of 160,000 and a frictional ratio of 1.6, extensively phosphorylated and partitioned totally into the aqueous phase. A comparison of the amino acid sequence of the chick HSP90 to that of the homologous HSP90 from yeast to man, reveals 64–96% identity respectively (Binart et al. 1989). The authors suggested that two hydrophilic regions A and B may play a role in the interaction of HSP90 with other proteins such as

steroid hormone receptors. In fact, the dimeric form of the HSP90 was confirmed and its structure was shown to be stabilized by hydrogen bonds (Radanyi et al. 1989). Furthermore, the cDNA-derived amino acid sequence of chick HSP90 revealed a "DNA like" structure: potential site of interaction with steroid receptors (Binart et al. 1989). The nucleotide sequence of a 2652 bp derived from a chicken HSP90 genomic clone was reported and two introns have been identified (Vourch et al. 1989). It was proven that HSP90 gene expression is constitutive and heat inducible. In the chick oviduct cells, HSP90 was located in the cytoplasm as aggregates, often inside small vesicles, while in the apical part of the cell, HSP90 was located at the Golgi complex (Pekki 1991). The epithelium also exhibited some cells with high levels of HSP90. It is interesting to note that HSP90 is associated with both microtubules and microfilaments (Czar et al. 1996). In fact, C-terminal half of HSP90 contains a sequence which is responsible for the cytoplasmic localization of the protein and the cytoplasmic anchoring signal is located between amino acids 333 and 664 (Passinen et al. 2001). It was shown that in contrast to HSP70, the 35S metabolically-labelled HSP90, which accumulates in the cytosoluble fraction 6–8 h after serum treatment, is not preferentially translocated to the nuclear compartment, although a small fraction is always present in the nucleus (Jérôme et al. 1993). It was also demonstrated that serum- or insulin-induced accumulation of HSP90α mRNA results from an activation of gene transcription and that HSP90α promotor activity is induced approximately fivefold after serum stimulation. Therefore, chicken HSP90 constitutively expressed in most cells, is up-regulated by thermal stress and by developmental and mitogenic stimuli. Indeed, a transient induced expression of the HSP90α gene takes place at both the messenger RNA and the protein synthesis level. This response is protein synthesis dependent and DNA synthesis independent. A possible link between cell cycle and HSP90α regulation was suggested (Jérôme et al. 1991).

It seems likely that the HSP90 alpha and beta genes are the result of a gene duplication event that occurred at the time of the emergence of vertebrates (Meng et al. 1993). Furthermore, avian HSP90β mRNA is not inducible by thermal stress or mitogenic stimuli, contrary to the mouse and human HSP90 alpha and beta mRNAs. Indeed, chicken HSP90β is the only vertebrate HSP90 insensitive to heat shock and there are some specific features of HSP90 beta gene structure and location explaining why chicken HSP90 beta mRNA is generally less abundant than alpha and is not inducible by heat shock or serum/growth factor stimulation (Meng et al. 1995).

The importance of ATP binding and hydrolysis by HSP90 in formation and function of protein heterocomplexes was shown (Grenert et al. 1999). Chicken HSP90 hydrolysing ATP activity was found to be 10–100-fold lower than that in yeast HSP90 and TRAP1, an HSP90 homologue found in mitochondria (Owen et al. 2002). The authors showed that sequences within the last one-fourth of HSP90 regulate ATP hydrolysis. The N-terminal ATP binding domain of HSP90 is necessary and sufficient for interaction with estrogen receptor (Bouhouche-Chatelier et al. 2001).There are two sites in HSP90 binding ATP. In fact, HSP90 N-terminal domain has a nonconventional nucleotide binding site and HSP90 possesses a second ATP-

binding site located on the C-terminal part of the protein (Garnier et al. 2002). HSP90 chaperone activity was shown to require the full-length protein and interaction among its multiple domains, indicating that the cooperation of multiple functional domains is essential for active, chaperone-mediated folding (Johnson et al. 2000).

The expression of HSP90 increased in the heart, liver and kidney of broilers after exposure to increased temperature for 2 h (Lei et al. 2009). In the heart and kidney, HSP90 mRNA transcription levels exhibited the same trend as the protein expression of HSP90. Induction of HSP90 mRNA and HSP90 protein at an early stressing stage indicated that heat stress can directly stimulate and quickly initiate the transcription of HSP90 mRNA and translation of HSP90 protein to protect cells. The HSP90α gene is shown to play an evolutionarily conserved role during somitogenesis in vertebrates in addition to providing protection to all cells of the embryo following stress (Sass and Krone 1997).

It was suggested that HSP90 can participate directly in the function of a broad range of cellular signal transduction components, including retinoid receptor signal transduction (Holley and Yamamoto 1995). In eukaryotic cells, HSP90 is associated with several protein kinases and regulates their activities. HSP90 was also reported to possess an autophosphorylase activity (Kim et al. 1999). In fact, chicken HSP90 participates in folding and stabilization of signal-transducing molecules including steroid hormone receptors and protein kinases and both amino- and carboxyl-terminal domains of HSP90 interact to modulate chaperone activity (Marcu et al. 2000). Depletion of HSP90β induces multiple defects in B cell receptor signaling (Shinozaki et al. 2006). Indeed, inhibition of HSP90 with geldanamycin resulted in the inactivation of MAPK/ERK and PI3K/AKT pathways leading to significantly reduced levels of IFN-γ, IL-6 and NO mRNAs in avian macrophages (Bhat et al. 2010). Therefore, in contrast to mammals, HSP90α but not HSP90β may play a major role in CpG ODN(2007) induced immunoactivation in avian macrophage cells. Collectively, these observations strongly suggest that signaling roles of HSP90 in avian species need further investigation. Recently, four novel members of the 90 kDa heat shock protein (HSP90) family expressed in Japanese quail, Coturnix japonica have been described (Nagahori et al. 2010). The coding regions of the genes, CjHSP90AA1, CjHSP90AB1, CjHSP90B1 and CjTRAP1, exhibited more than 94% similarity to their related genes in chicken. Furthermore, CjHSP90AA1 exhibited a robust response to heat shock treatment.

5.1.2.3 Heat Shock Protein 32 (HSP32) (HO-1)

HO-1 is the stress-inducible isoform of the three HO isoforms described to date, serving as a critical protective mechanism in vertebrate systems responsible for adaptation to oxidative, inflammatory, and cytotoxic stress (Wu et al. 2011; Fredenburgh et al. 2015). In fact, HO-1 (32 kDa), also known as heat shock protein-32 (HSP32), is shown to be expressed at a relatively low level in most tissues. It is proven that HO-1 is endoplasmic reticulum phase II enzyme catalysing the

rate-limiting step in heme degradation, producing free iron (Fe^{2+}), carbon monoxide (CO) and biliverdin (Soares and Bach 2009). Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. It is interesting to mention that the products of the aforementioned reaction can trigger signalling cascades leading to improvement of antioxidant defences and protection against oxidative stress. In particular, CO can modulate the production of proinflammatory or anti-inflammatory cytokines and mediators having immunomodulatory effects with respect to regulating the functions of antigen-presenting cells, dendritic cells, and regulatory T cells (Ryter and Choi 2016). It seems likely that products of the HO-1 reaction namely CO and biliverdin have also cytoprotective, anti-inflammatory and anti-apoptotic properties in stress conditions (Durante 2010; Haines et al. 2012; Zahir et al. 2015). Cells exposed to low concentrations of CO were shown to respond by an increase in ROS formation (e.g. oxidative conditioning) with important consequences for inflammation, proliferation, mitochondria biogenesis, and apoptosis (Bilban et al. 2008). Actually, the degradation of heme by HO-1, the signaling actions of CO, the antioxidant protective action of biliverdin/bilirubin, and the sequestration of Fe^{2+} by ferritin are suggested to contribute to the anti-inflammatory effects of HO-1 (Pae and Chung 2009) and increase stress resistance. Furthermore, recent studies have demonstrated that HO-1 inhibits stress-induced extrinsic and intrinsic apoptotic pathways in vitro (Morse et al. 2009). The vital importance of HO-1 in stress adaptation have been confirmed in HO-1-deficient mice models showing atypical proinflammatory immune response (Kapturczak et al. 2004) with increased vulnerability to endotoxin sepsis (Poss and Tonegawa 1997), defective expression of interferon-γ (Tzima et al. 2009) and increased susceptibility to apoptosis (Vachharajani et al. 2000; True et al. 2007). Morover, HO-1 knockout mice were characterised by very low survival (~1%–5% of litters) and high levels of oxidative stress with a shortened life span (Wegiel et al. 2014a, b). In fact, HO-1 knockout mice were shown to be extremely sensitive to oxidative stress caused by ischemia and reperfusion (Yet et al. 1999; Liu et al. 2005) and to develop anemia associated with hepatic and renal iron overload leading to oxidative tissue injury and chronic inflammation (Poss and Tonegawa 1997). The aforementioned observations provide substantial evidence to support the implication of HO-1 in stress response.

The half-lives of HO-1 mRNA and protein are shown to be approximately 3 hours and 15–21 hours, respectively (Dennery 2000). In humans, the HO-1 gene (Hmox1) is located on chromosome 22q12 and consists of four introns and five exons. The regulatory region of the mammalian HO-1 gene has a promoter, a proximal enhancer, and two or more distal enhancers (for review see Schipper and Song 2015). The Hmox1 promoter is shown to exhibit a range of binding sites (for AP-1, AP-2, NF-κB, and HIF-1), as well as HSE sequences, metal response elements and stress-response elements. Therefore, the complex gene structure explains its high sensitivity to induction by diverse pro-oxidant and inflammatory stimuli including heme, dopamine, TNF-α, IL-1β, cysteamine, β-amyloid, H2O2, hyperoxia, UV light, heavy metals, lipopolysaccharide, etc. (Schipper and Song 2015). In vertebrates HO-1 is shown to be upregulated by its substrate heme as well as by a wide variety of stressors including heavy metals, heat shock, ischemia, ROS, RNS, bacte-

rial endotoxins, radiation, hypoxia, H2O2, nitric oxide, etc. (Chang et al. 2009; Wegiel et al. 2014a, b). Furthermore, inflammatory mediators such IL-1, TNF-α, LPS are also shown to upregulate HO-1 in vitro (Terry et al. 1998; Niess et al. 1999).

At the cellular level, HO-1 is highly expressed in the organs participating in degrading senescent red blood cells, including spleen, reticuloendothelial cells of the liver and bone marrow (Immenschuh et al. 1999) as well as in macrophages (Bissell et al. 1972) and dendritic cells (Chauveau et al. 2005). In fact, HO-1 upregulation in various cells is shown to attenuate the expression of various proinflammatory genes (Lee and Chau 2002; Wijayanti et al. 2004). Furthermore, HO-1 is of great importance for building immunocompetence. Indeed, induction of HO-1 in dendritic cells alters their maturation state and interaction with other cells (Chauveau et al. 2005; Remy et al. 2009), including T lymphocytes (George et al. 2008; Moreau et al. 2009) and macrophages (Wegiel et al. 2014a, b; Nakamichi et al. 2005; Choi et al. 2010).

Regulation of HO-1 activity as an adaptive response to stress is mediated via several key initiator and feedback control processes. In particular, the transcriptional regulation of the HO-1 gene is shown to be attributed to several transcription factors including Nrf2, Bach1 (Igarashi and Sun 2006; Jang et al. 2009), HIF-1 (Semenza 2010) and PPARs (Ndisang 2014). It seems likely that MAPK signaling is involved in HO-1 induction (De Backer et al. 2009). In particular, the anti-inflammatory cytokine IL-10 was shown to induce HO-1 expression via a p38 MAPK-dependent pathway (Lee and Chau 2002). Indeed, the antiapoptotic effect of CO was shown to be mediated by the activation of the p38 MAPK signal transduction pathway and required the activation of the transcription factor NF-κB (Soares et al. 2002). Furthermore, the phosphatidylinositol-3 kinase (PI3K)/Akt signaling also modulates HO-1 activity (Salinas et al. 2004). In addition, HO-1 is involved in suppression of the expression of the pro-inflammatory cytokine TNF-α, while an HO-1 inhibitor (zinc protoporphyrin) attenuated this effect (Lee and Chau 2002). Furthermore, HO-1 is an important regulator of cellular metabolism, and its activity may affect NADPH- and oxygen-consuming pathways, including fatty acid synthesis, oxidative metabolism of cytochrome p450, or modulation of ROS generation in phagocytes (Wegiel et al. 2014a, b).

5.1.2.3.1 Chicken HO-1

Data on HO-1 expression and its protective actions in poultry production are very limited. In early 1990th, HO-1 was purified from liver microsomes of chicks pretreated with cadmium chloride (Bonkovsky et al. 1990). The molecular weight of the enzyme was shown to be 33,000 Da and the pH optimum of the reaction was 7.4. It was also shown that Hg^{2+} inhibited HO-1 activity by 67% at 10 μM and totally at 15 μM. Comparison of sequences to those derived from cDNA sequences for the major inducible rat and human HO-1 showed 69% and 76% similarities, respectively (Bonkovsky et al. 1990). Next year, a cDNA from a chick liver library that

encodes for HO-1 has been cloned and sequenced (Evans et al. 1991). The protein corresponding to this fragment of DNA was found to compose of 296 amino acid residues and has a molecular mass of 33,509 Da. The similarity of chick HO-1 to rat and human HO-1 (nucleotides 66% and amino acids 62%) was confirmed to be moderately high. It was also shown that Cd-dependent induction of HO-1 was due to increased transcription of the gene or stabilization of its message (Evans et al. 1991). Similar to mammalian HO-1, chicken HO-1 has five exons and four introns (Lu et al. 1998). In the DNA sequence there are consensus sequences corresponding to numerous transcription factor recognition elements, including AP-1, AP-2, NF-kB, C/EBP, c-Myc and a metal-responding element identified in the promoter region (Lu et al. 1998). Furthermore, chick HO-1 promoter region responded to sodium arsenite, H2O2 and transition metals, but not to heme. The chick HO-1 promoter region also contains a unique sequence that localized at −3.7 kb upstream of the transcription start site of the chick HO-1 gene and subserves up-regulation of the gene by metalloporphyrins (Shan et al. 2002; 2004). Furthermore, the chick HO-1 promoter region was shown to contain "expanded" by three base pairs AP-1 sites that are important for up-regulation of the gene by heme and cobalt protoporphyrin, but not other metalloporphyrins (Shan et al. 2004).

HO-1 could be detected in microsomes from all chick or rat organs studied, including spleen, testis and brain (Greene et al. 1991). The effects of heme on the induction of mRNA and protein synthesis for HO-1 have been studied in primary cultures of chick embryo liver cells (Cable et al. 1993). It was shown that heme increased (up to 20-fold) the amount of mRNA and the rate of HO-1 gene transcription in a dose-dependent fashion. In fact, 7–15 h after heme addition, the half-life of HO-1 mRNA was 3.5 h in the presence or absence of actinomycin D, while the half-life of heme-induced HO-1 protein was 15 h (Cable et al. 1993). Similarities were observed with respect to regulation of HO-1 expression in primary chick embryo hepatocytes and chicken hepatoma cells (Gabis et al. 1996). It seems likely that HO-1 synthesis is under hormonal control. For example, the effects of various hormones on the induction of HO-1 in monolayer cultures in chick embryo hepatocytes were examined (Sardana et al. 1985). Indeed, insulin is shown to suppress the activity of basal as well as Co^{2+}-induced HO-1, while hydrocortisone suppressed the basal enzyme activity and slightly enhanced Co^{2+} −induced enzyme activity. In contrast, triiodothyronine caused a slight increase of both uninduced and induced levels of the enzyme (Sardana et al. 1985).

There is a range of in vitro studies, mainly with embryonic chick cells, to address possible mechanisms of HO-1 induction by various metals. For example, in primary cultures of embryonic chick liver cells HO-1 activity was shown to be upregulated by inorganic cobalt (Maines and Sinclair 1977). Treatment of isolated chick embryo liver cells in vitro with sodium arsenite or melarsoprol also showed a potent induction of HO-1 (Sardana et al. 1981). In monolayer cultures of chick embryo liver cells the most potent HO-1 inducing action was exhibited by Co^{2+}, Cd^{2+}, Sb^{3+}, As^{3+}, and Au^{1+} followed by lower induction observed with $Cu^{2+,}$ Fe^{2+}, and Fe^{3+}(Sardana et al. 1982). In contrast, adding Zn^{2+} (20 μM), Mn^{2+} (50 μM) or cysteine (400 μM) to Co^{2+}-treated cells blocked/inhibited the HO-1 induction. It seems likely that

increased HO-1 activity by metal treatment is dependent on fresh RNA and protein synthesis since cycloheximide and actinomycin D blocked the induction of HO-1 (Sardana et al. 1982). The activity of HO-1in chick embryo is shown to be enhanced by cadmium chloride treatment (Prasad and Datta 1984). It has been suggested that induction of HO-1 by drugs and metals occurs by different mechanisms. For example, a drug phenobarbitone induced HO-1 by increasing hepatic haem formation, while increases in HO activity by metals (cobalt, cadmium or iron) were not dependent on increased haem synthesis and were not inhibited by 4,6-dioxoheptanoic acid (Lincoln et al. 1988). In cultured chick embryo liver cells, synergistic induction of HO-1 by iron, added with the phenobarbital-like drug, glutethimide was heme-dependent (Cable et al. 1990). Addition of an inhibitor of heme biosynthesis abolished the synergistic induction of heme oxygenase providing evidence for the heme-dependent mechanism of induction. Both HO-1 mRNA and protein levels were shown to correlate with changes in HO-1 activity indicating that glutethimide and iron induce HO-1 at the transcriptional level. Induction of the HO-1 gene by heme is shown to be fundamentally different from that produced by transition metals or sodium arsenite and expression of the HO-1 gene is highly conserved across species (Lu et al. 1997). Notably, in chick embryo liver cell cultures, HO-1 responded to sodium arsenite treatment in a dose-dependent fashion, and the response was rapid and transient. Although 2.5 μM arsenite is shown to induce HO-1 four- to six-fold, this had no effect on degradation of exogenous heme (Jacobs et al. 1999).

It seems likely that similar to mammals, in birds HO-1 induction in stress conditions is mediated by various signaling pathways. For example, in chicken hepatoma cells, MAP kinases ERK and p38 are shown to be involved in the induction of HO-1, and at least one AP-1 element is involved in this response (Elbirt et al. 1998). In particular, it was shown that the phenylarsine oxide (PAO), an inhibitor of protein tyrosine phosphatases, upregulated HO-1 gene activity in dose- and time-dependent fashion and both an AP-1 element and a metal responsive element were involved in the PAO-mediated induction of the HO-1 activity (Shan et al. 1999). Indeed, a short (1–15 min) exposure of normal hepatocytes to low concentrations (0.5–3 μM) of PAO are shown to produce a marked increase in mRNA and protein of HO-1, which occur without producing changes in cellular glutathione levels or stabilization of HO-1 message (Gildemeister et al. 2001). Furthermore, preincubation of cells with inhibitors of protein synthesis decreased the ability of PAO to increase levels of HO-1 mRNA, suggesting that the inductive effect requires de novo protein synthesis. Addition of thiol donors abrogated the PAO-mediated induction of HO-1 in a dose-dependent fashion. Addition of genistein, a tyrosine kinase inhibitor, blunted the induction produced by both PAO and heme (Gildemeister et al. 2001). It was shown that induction of the chicken HO-1 gene by sodium arsenite or cobalt chloride is mediated through oxidative stress pathway(s) by activation of AP-1 proteins (Lu et al. 2000). It seems likely that vascular endothelial growth factor upregulates HO-1 protein expression in vivo in chicken embryo chorioallantoic membranes by a mechanism dependent on an increase in cytosolic calcium levels and activation of protein kinase C (Fernandez and Bonkovsky 2003).

In chick embryo hepatocytes heme breakdown occurred predominantly, if not solely, by heme oxygenase (Lincoln et al. 1989). It seems likely, that increased HO-1 expression in chicken embryos between internal (day 19) and external pipping (day 20) (Druyan et al. 2007) is an adaptive mechanism responsible for increased protection of tissues during this stressful period of the ontogenesis. Similarly, increased concentrations of vitamin E and carotenoids were observed in chicken embryonic tissues at the same period of time (Surai 2002), providing an effective protection at hatching. It is well known, that various phytochemicals can affect HO-1 activity (Barbagallo et al. 2013; Murakami 2014), however, more research is needed to understand molecular mechanisms of their interactions. For example, sulpharaphane containing broccoli extract and four different essential oils were tested in the 2 week old broilers as feed additives for 3 weeks. The phytogenic feed additives increased HO-1 activity in the jejunum, but decreased it in the liver (Mueller et al. 2012). It is interesting to note that relative mRNA expression of HIF-1 (heart) was increased and HO-1 (heart and liver) was decreased at week 4 in broilers fed with high ME and protein diet (Peng et al. 2013). From the aforementioned data it is clear that HO-1 is well characterised in avian species, however, its response to different stresses in commercial and wild birds are still not fully characterised.

Thus, an analysis of the published data leads to the conclusion that HSP play a significant role in cell/organism protection against various stresses being an integral part of the antioxidant network responsible for proteostasis maintenance.

5.1.3 Practical Applications of HSP Expression in Poultry Production

5.1.3.1 Heat Stress and HSP in Avian Species

The university of the HSR and conservation of proteins induced by heat stress were shown in experiments with various species. As mentioned above, effect of heat stress on the expression of HSP in avian species started in early 1980th (Kelley and Schlesinger 1982; Voellmy and Bromley 1982; Voellmy et al. 1983). Similarly, exposure of chick myotube cultures to an increased temperature (45 °C) caused extensive synthesis of three major HSP (25 kDa, 65 kDa and 81 kDa). When experimental cells were allowed to recover from heat-shock treatment at 37 °C for 6–8 h, HSP synthesis declined to levels comparable to those in control cultures maintained at 37 °C (Bag et al. 1983a, b). Therefore, four major chicken stress mRNAs coding HSP with apparent molecular weights of 88 kDa, 71 kDa, 35 kDa and 23 kDa were separated and their properties were studied (White and Hightower 1984). Exposure of the 11-day embryonic chicken lens to elevated temperature (45 °C) dramatically increased the synthesis of three HSP with subunit molecular weights of 89,000, 70,000 and 24,000 Da. Furthermore, the functional half-lives at 37 °C of the mRNAs encoding the lens HSP were about 3–5 hr (Collier and Schlesinger 1986a).

The intracellular distributions of the major heat shock proteins, HSP89, HSP70, and HSP24 were studied in chicken embryo fibroblasts stressed by heat shock, allowed to recover and then restressed (Collier and Schlesinger 1985b). It was shown that HSP89 was localized primarily to the cytoplasm and during the restress a portion of this protein was associated with the nuclear region. In contrast, significant amount of HSP70 was shown to move to the nucleus during stress. In general, the nuclear HSP reappeared in the cytoplasm in cells allowed to recover at normal temperatures. It is interesting to note that, sodium arsenite also induces HSP and their distributions were similar to that observed after heat shock, except for HSP89, which remained cytoplasmic (Collier and Schlesinger 1986b). Reticulocytes, purified from the blood of quail and chickens responded to heat shock by the synthesis of HSP90, HSP70 and HSP26 (quail) or HSP24 (chicken) and the depressed synthesis of many other proteins normally produced at a physiological temperature (Atkinson et al. 1986). It was shown that the expression of each protein depended upon the particular temperature and duration of heat exposure. It was noted that HSP70 was constitutively synthesized and selectively partitioned between cellular compartments. Furthermore, heat shock induced synthesis of the HSP90, HSP70 and HSP26 in quail was prevented by actinomycin D (Atkinson et al. 1986).

Heat shock response is a universal biological protective mechanism in stress conditions. Indeed, cultured bovine, equine, ovine and chicken lymphocytes responded to heat stress by the increased synthesis of HSP. In particular, HSP70 and HSP90 were synthesized in all species and induction time of the HSP synthesis comprised 30–60 minutes (Guerriero and Raynes 1990). Heat shock response is an important mechanism of immune cells protection. Actually, heat-induced chicken macrophages synthesized HSP23, HSP70 and HSP90. The optimal temperature and time for induction of these HSP was 45–46 °C for 1 h, with a variable recovery period for each HSP (Miller and Qureshi 1992a). A comparison of HSP synthesis among peritoneal macrophages (PM) from chickens, turkeys, quail, and ducks shows the highly conserved nature of heat-shock response within birds. In fact, macrophage cultures from each avian species expressed the three major HSP (HSP23, HSP70 and HSP90) following heat-shock exposure (1-h heat shock at 45 °C) (Miller and Qureshi 1992b). There was also increased expression of a new HSP called P32, which probably was HSP32 (known as HO-1) in all 4 species. The authors also showed that the duck P32 and HSP23 were lower in molecular mass than their respective homologues expressed in chickens, turkeys, and quail macrophage cultures indicating some species-specific differences between HSP in avian species (Miller and Qureshi 1992b). Chicken macrophages (mononuclear phagocytic cell line MQ-NCSU) exposed to LPS under control (41 °C) temperatures expressed enhanced synthesis of classical HSP23, HSP70, and HSP90, as well as heat-inducible 32-kDa protein (P32), and a novel LPS-induced 120-kDa protein (P120). In comparison to LPS treatment, MQ-NCSU cells exposed to 45 °C (HS) expressed HSP23, HSP70, HSP90, and P32 but not P120 (Miller and Qureshi 1992c). It is interesting to note that lead acetate caused similar upregulation of the same four HSP (HSP23, HSP70, HSP90 and P32) previously expressed by macrophages after in vitro and in vivo heat treatment (Miller and Qureshi 1992d). It seems

likely that various nutritional deficiencies could affect HSP response. For example, during acute in vivo heat stress, a HSP response was not inducible in chickens deficient in inorganic phosphorus (Edens et al. 1992) and they were more susceptible to heat stress.

Increased HSP expression in response to various stresses, including heat stress, is shown to be a universal mechanism in various chicken tissues. For example, both the amount and polyadenylation of HSP70 and ubiquitin transcripts increased when male germ cells from adult chicken testis were exposed to elevated (46 °C) temperatures (Mezquita et al. 1998). Similarly, there was a marked increase in HSP70 expression in the brains of female broiler chickens after 4 days (from d35 to d38) of heat treatment (38+/−1 degrees C for 2 h/d; Zulkifli et al. 2002). In addition, in chicken pineal cells several heat shock proteins (HSP 25, 70, and 90) are shown to be synthesized under temperature conditions (Wolfe and Zatz 1994). Thermal stress (41 °C) caused induction of HSP90α and HSP90β in chicken heart, liver and spleen, but HSP90α and HSP90β mRNA levels were stable in brain. Transcription of HSP70 also increased in all organs from chickens in heat stress groups when compared to chickens in control groups (Mahmoud et al. 2004a). The elevation of the three HSP in heart, may act as protective mechanism in adverse environments. For example, three main chicken HSP (HSP60, HSP70, HSP90), and their corresponding mRNAs in the heart tissue of heat-stressed (37 °C for 2–10 hours) broilers, elevated significantly after 2 h of heat exposure and decreased quickly with continued heat stress. However, the level of HSP60 protein in the heart increased and maintained throughout heat exposure (Yu et al. 2008). Indeed, there is a great diversity in heat shock response in different tissues. For example, thirty two week old broiler breeders were subjected either to acute (step-wisely increasing temperature from 21 to 35 °C within 24 hours) or chronic (32 °C for 8 weeks) high temperature exposure. There was a tissue specificity in the response to acute and chronic stress (Xie et al. 2014). For example, in the heart, acute heat challenge increased lipid peroxidation and upregulated gene expression of all four HSFs. Furthermore, during chronic heat treatment, the HSP 70 mRNA level was increased and HSP 90 mRNA was decreased. At the same time, in the liver, protein oxidation was alleviated during acute heat challenge and gene expression of HSF2, 3 and 4 and HSP70 were highly induced. In addition, HSP90 expression was increased by chronic thermal treatment. In the muscle, both types of heat stress increased protein oxidation, but HSFs and HSP gene expression remained unaltered and only tendencies to increase were observed in HSP70 and HSP90 gene expression after acute heat stress (Xie et al. 2014). The expression of HSP27, HSP70, and HSP90 mRNA in the bursa of Fabricius and spleen of 42-d old chickens were increased due to heat stress (37 ± 2 °C for 15 d; Liu et al. 2014). However, under the same stress conditions the expression of HSP27 and HSP90 mRNA in thymus were decreased. In testis of heat-stressed cockerels (38 °C for 4 hours) the heat shock proteins, chaperonin containing t-complex, and proteasome subunits were downregulated (Wang et al. 2014). Therefore, acute heat stress impairs the processes of translation, protein folding, and protein degradation resulting in apoptosis and spermatogenesis disturbance. Heat stress in 21-day-old broilers was associated with up-regulation of the rectal temperature and the mRNA

expression of HSP70 in the liver (Zuo et al. 2015). Heat stress (40 °C for 2 h) in the growing chickens (41 day old) caused significant increases in sera corticosterone, LDH, MDA and SOD, the expression of HSP90 and HSP70 in the pectoralis major. Furthermore, HSP90 was shown to positively correlate with corticosterone and SOD activities (Hao and Gu 2014). In chicken hypothalamus the transcripts of HSP90 decreased while HSP40 increased in response to thermal stress (34 °C for 24 h; Sun et al. 2015a).

It seems likely that gene expression changes due to heat stress are of great importance for cell adaptation to stress. For example, heat stress (38 °C for 4 h) was associated with upregulation of 169 genes and downregulation of 140 genes in rooster testis (Wang et al. 2013). Differentially expressed genes were mainly related to response to stress, transport, signal transduction, and metabolism. Indeed, HSP genes (HSP25, HSP70 and HSP90AA1) and related chaperones were the major upregulated groups in chicken testes after acute heat stress. Heat stress in chickens was associated with 166 differentially expressed genes in the brain, 219 in the leg muscle and 317 in the liver (Luo et al. 2014). Six of these genes were differentially expressed in all three tissues and included heat shock protein genes (HSPH1-heat shock 105 kDa/110 kDa protein 1 and HSP25), apoptosis-related genes (RB1CC1, BAG3), a cell proliferation and differentiation-related gene and the hunger and energy metabolism related gene. Various functional clusters were related to the effects of heat stress, including those for cytoskeleton, extracellular space, ion binding and energy metabolism (Luo et al. 2014). It seems likely that HSP expression in response to increased temperature is a universal cellular mechanism protecting proteins against unfavourable changes, including misfolding and molecular mechanisms of HSR need further research.

5.1.3.2 Thermal Manipulation and HSP

It has been noted that exposure of cells to non-lethal elevations in temperature activates cellular stress responses and induces a state of thermotolerance, characterised by increased cell resistance to subsequent lethal insults. Indeed, preconditioning is a phenomenon in which a prior stress provides protection against a subsequent and more severe stressful exposure/dose (Calabrese et al. 2015a, b; 2016), probably, via hermetic mechanisms. In fact, in early 1980th the idea of thermotolerance received substantial attention. In particular, in studies with Chinese hamster ovary cells thermal tolerance was shown to be developed during chronic or acute heating. For example, cells that expressed thermal tolerance as a result of a chronic heat treatment at 42 °C also expressed thermal tolerance to a subsequent acute treatment at 45.5 °C (Spiro et al. 1982). Also, cells heated acutely showed tolerance to chronic hyperthermia. Therefore when cells are tolerant to chronic hyperthermia they are also tolerant to acute hyperthermia and that the reverse is also true.

Physiological studies with wild birds and observations on the natural incubation of eggs by domestic hens indicate that egg incubation temperature has a great fluc-

tuation (Webb 1987) and it was hypothesized that this could have a positive effect on adaptive ability of chickens (Decuypere 1984; Minne and Decuypere 1984). Therefore, exposing embryos to periods of high or low temperature during incubation could potentially affect their thermotolerance reflecting the programming effect of early development on the subsequent performance of chicken. Indeed, increasing the incubation temperature during important stages of the embryo development, associated with thermoregulation and stress, was shown to be an effective and long-lasting means of acquiring thermotolerance in growing chickens reared in cages (Piestun et al. 2008, 2009). Similarly, exposure of chicken embryo to a high incubation temperature (39.5 °C for 12 h/d between E7 to E16) reduced abdominal fat pad by 8% and increased breast muscle yield (Loyau et al. 2013), and improved FCR (Piestun et al. 2013). Recently it has been shown that, an increasing incubation temperature during early embryogenesis positively influences the growth and carcass traits of the broilers, accompanied with a partly negative impact on meat quality (drip loss, shear force, lightness; Janisch et al. 2015). It is interesting to note that the growth effects were sex-dependent, as significant weight differences could be only found in female broilers. Furthermore, it was concluded that thermal manipulation (TM) during turkey embryogenesis might have altered the thermoregulatory set point, and thus lowered the embryo metabolic rate, which might have a long-lasting posthatch effect (Piestun et al. 2015a). In fact, during the first week posthatch, myoblast proliferation activity was significantly higher in TM groups compared to controls; however, at 2 wk of age it was significantly lower (Piestun et al. 2015b). Therefore, TM of the chick embryo has been suggested to improve the ability of the chicks to reduce their heat production during thermal stress later in their life. Furthermore, TM was shown to affect embryo physiology, growth, meat yield and processing quality (Moraes et al. 2004; Yahav et al. 2004a, b; Collin et al. 2005, 2007) as well as heat tolerance, associated with lower body temperature until slaughter age (Piestun et al. 2008, 2009).

However, the data on the effect of TM on thermotolerance published to date are not consistent and several studies reported no effect. Indeed, short periods 1 or 2 °C changes in incubation temperature did not influence hatching efficiency of broilers (Yahav et al. 2004a, b; Collin et al. 2007; Tona et al. 2008; Yalçın et al. 2008, 2012) or laying chicks (Walstra et al. 2010). For example, a higher egg shell temperature within the first embryonic week increases the weight of 21.5 d old broiler embryos but not of 7 d old chicks (Lourens et al. 2005). Similarly, incubation at higher temperatures between ED 7 and 10 resulted in comparable weights of broilers at day 36 post-hatch (Werner et al. 2010). Lower carcass yields of 33 d old broilers and lower live weights and leg weights of 69 d old birds was reported after increasing incubation temperature (+1 °C) between ED 3 and 6 compared to embryos at the normal incubation temperature (Krischek et al. 2013). In general, there was no negative effect of higher or lower temperatures from day 10 to 18 of incubation on hatching performance and hatch weight of laying chicks and prenatal temperature conditioning of laying hen embryos had no advantage on laying performance of hens under temperature stress conditions (Kamanli et al. 2015). Principally, window of thermal manipulation of the chicken embryo is quite narrow since eggshell temperature

manipulations (38.4- to 39.0 °C for three hours daily) applied during hatching term (days 19 to 21) negatively affected incubation results and broiler performance, especially mortality due to ascites (Sozcu and Ipek 2015).

It seem likely that inconsistency of the results of TM during avian embryonic development reflects lack of understanding molecular mechanisms of the acquisition of TM. Therefore, even small variations in experimental set up (breed, sex, temperature as well as time and duration of its application, etc.) could substantially affect experimental results. There is no doubt that TM during embryogenesis has long-lasting effects on physiology, which may potentially modulate gene expression and metabolism in peripheral tissues being a background for an adaptive mechanism such as heat tolerance (Loyau et al. 2013). Without affecting hatchability, TM resulted in lower body temperature at hatching and until d 28 post-hatch and significant changes in plasma thyroid hormone concentrations. Notably, fine tuning of incubation conditions, taking into account the level and duration of increases in temperature and relative humidity during a critical period of embryogenesis is considered to be a powerful tool for improvement thermotolerance (resistance to environmental changes) and growth and development of the posthatch chicks (Loyau et al. 2015). It seems likely that the induction of epigenetic mechanisms related to control body temperature provides long lasting effect in postnatal development of birds. In fact, DNA methylation and histone modification patterns during the late embryonic and early postnatal development of chickens have been recently described (Li et al. 2015) and it would be important to study effects of TM on these epigenetic-related processes.

Recently the effects of increasing the incubation temperature by 1 °C from day 11 to 20 on the embryonic and posthatch skeletal muscle development of the Peking duck were investigated and gene expression profile of leg muscle tissues from thermally manipulated ducks was assessed (Liu et al. 2015b). Indeed, altering the incubation temperature had immediate and long-lasting effects on phenotypic changes in the embryonic and post-hatching muscle development. In particular, expression levels of total 1370 genes were altered in muscle tissues by the thermal treatments. In fact, cellular processes including metabolism, cell cycle, catalytic activity, and enzyme regulatory activity may have involved in the muscle mass impacted by thermal manipulation (Liu et al. 2015b). The transcriptome studies confirmed the complexity of the heat stress response. In fact, heat stress responsive genes in the chicken male white leghorn hepatocellular (LMH) cell line have been identified (Sun et al. 2015a, b). The transcripts of 812 genes were shown to respond to heat stress with 235 genes upregulated and 577 downregulated following 2.5 h of heat stress. Genes whose products function as chaperones, as well as genes affecting collagen synthesis and deposition, transcription factors, chromatin remodelers, and genes modulating the WNT and TGF-β pathways were upregulated. At the same time, genes affecting DNA replication and repair along with chromosomal segregation were found to be predominant among the downregulated genes (Sun et al. 2015a, b).

As mentioned above, molecular mechanisms of acquired thermotolerance are not fully understood but HSP expression and synthesis are thought to play an essential

part in this process. Initial work on effect of early chick exposure to heat stress on the HSP expression and thermotolerance in later life started at the end of 1990th. In fact, when chicks at early age were exposed to heat (conditioning; 36 °C for 24 h at age of 5 days) to improve thermotolerance, lower levels of HSP induction in the heart and lung tissues was observed in the treated chickens (Yahav et al. 1997a). It was shown that the induction of HSP in the heart and lung tissues of the whole animal correlates with the body temperature. Indeed, at the age of 42 days, challenge with acute heat stress (35 °C) resulted in a large increase in cloacal temperature of the control chickens and by a more moderate increase in the conditioned chickens. Mortality during the thermal challenge was significantly higher in the control chickens than in the conditioned ones. However, the synthesis rate of HSP70 and HSP90 during the first hour of heat challenge, accelerated gradually in control chickens, whereas in the conditioned chickens it accelerated only after 3 hours and in a more moderate response (Yahav et al. 1997b). The authors suggested that HSP response does not represent a part of the long-term mechanism that is evoked by the early age conditioning. However, next year that conclusion was challenged. In fact, heat conditioning (41 °C for 1 h, daily) in both broiler chickens and turkey poults enhanced expression of HSP90, HSP70, and HSP23 in peripheral leukocytes when these cells were heat stressed in vitro (Wang and Edens 1998). In chickens, 1wk conditioning was sufficient to enhance the HS response when the leukocytes were heat stressed in vitro in the following week. However, in turkey a 3-wk heat conditioning period, followed by a 2-wk rest period, was associated with maximal induction of the three HSP studied in these experiments. It is interesting to note that enhancement in HSP expression was evident for periods up to 4 wk. after termination of the daily heat conditioning episodes (Wang and Edens 1998). This was further developed in a study where resistance to acute heat stress (41 °C, days 39–42) and concentration of HSP70 were increased in chickens subjected to early heat stress during rearing (3 heat stress episodes at 35 °C for 4 h per week; Givisiez et al. 1999). Furthermore, a positive correlation was observed between HSP70 concentration and the time taken for a 3 °C increase in rectal temperature. It was shown that a combination of early feed restriction (days 4–6) and heat stress (36 °C for 1 h from day 1 to day 21, FRHT) was associated with better HSP70 response after heat stress (d 41). Furthermore, early stress improved weight gain and resistance to IBD in male broiler chickens under heat stress conditions (Liew et al. 2003). The authors attributed the improved heat tolerance and disease resistance in FRHT birds to better HSP 70 response. Ten years later the interest in role of HSP in chicken thermotolerance was renewed.

TM during embryogenesis (39 °C for 9–18 h) resulted in a significant increase in mRNA levels of HSP90, HSP60 and HSF1 in muscle, heart and brain tissues during embryogenesis and during thermal challenge (43 °C) at days 10 and 28 post-hatching (Al-Zghoul et al. 2015). The authors suggested that the changes in HSP90, HSP60 and HSF1 gene expressions could be associated with improved thermotolerance acquisition in TM chicks. However, a recent research from Rajkumar et al. (2015) showed that exposure to increased temperature (by 2 °C) during incubation (days 16–18) resulted in reduced expressions of HSP70 mRNA in various tissues

indicating better thermotolerance of the heat-exposed birds. It seems likely that temperature, duration of heat treatment, stages of embryo development and other differences in experimental set up could be responsible for variability in HSP response.

It seems likely that long-term results of TM of the avian embryo and thermotolerance acquisition are mediated not only by HSP, but other molecular mechanisms are also involved. For example, the role of HSP in acquisition of thermotolerance was questioned, since a major inducible HSP, HSP68, was not required for the development of thermotolerance in rat fibroblasts (Widelitz et al. 1986) or mouse plasma cytoma cells (Aujame and Firko 1988). Furthermore, it was demonstrated that P388D1, a mouse macrophage tumour cell line, failed to induce HSP in response to either heat stress (42 °C, 1 h) or to heavy metal stress induced by arsenic trioxide (5–20 μM), however, cells exhibited thermotolerance in the absence of induced HSP. The tolerance was shown to be abrogated in cells treated with cycloheximide (250 ng/ml) suggested that thermotolerance was dependent on de novo protein synthesis (Oommen et al. 2013). Furthermore, posttranslational histone modifications in the promoters of HSP80 and HSP90 are suggested to be involved in formation of heat-acclimation-mediated cytoprotective memory (Horowitz 2014). Indeed, there is a need for more research in this area to elucidate molecular mechanisms of thermotolerance with a specific emphasis to HSP and their interactions with various elements of antioxidant defence systems, including transcription factors.

5.1.3.3 Heavy Metals and HSP

Heat shock proteins are important cellular tools in protection against heavy metal toxicity. For example, splenocytes harvested in presence of sodium arsenite were characterized by increased expression of HSP70 and serum levels of HSP70 in broiler chicken also increased after continuous feeding of sodium arsenite in drinking water (Das et al. 2010). Similarly, the levels of HSP mRNA (HSP90, HSP70, HSP60, HSP40 and HSP27) and protein (HSP70 and HSP60) expression in immune organs of chickens were significantly increased in the As2O3 treatment groups compared with the corresponding control groups (Guo et al. 2016). However, HSP response depends on the toxicant dose used. For example, addition of lead to the chicken diet (200 mg lead acetate/kg diet) significantly decreased feed intake, body weight gain, and feed efficiency, upregulated the antioxidant enzymes gene expression together with the downregulation of glutathione S-transferase and HSP70 in the jejunum (Ebrahimi et al. 2015). In chicken spleen lymphocytes Mn had a dosage-dependent effect on HSP27, HSP40, HSP60, HSP70, and HSP90 mRNA expression in chicken spleen lymphocytes in vitro: the mRNA expression of the heat shock proteins was induced at lower concentrations of manganese and was inhibited at higher concentrations (Zhu et al. 2013). Therefore, as manganese concentration increased, the mRNA expression of the heat shock proteins first increased and then decreased.

5.1.3.4 Dietary AO and HSP

Since all antioxidants in the body are working together to build the effective antioxidant defence network, the increase concentration of one antioxidant can be associated with no need for increase another antioxidant element in stress conditions.

5.1.3.4.1 Vitamin E

Vitamin E is considered to be a main chain-breaking antioxidant in biological systems and its roles in poultry production are difficult to overestimate (Surai 1999b, 2002; 2014; Surai and Fisinin 2014). It was shown that vitamin E, added to the Vero cell culture prior mycotoxins (citrinin, zearalenone and T2 toxin) was able to prevent an induction of HSP70 expression due to mycotoxins (El Golli et al. 2006). In isolation-stressed quail, vitamin E or vitamin C were shown to prevent an increase in HSP70 expression in the brain and heart (Soleimani et al. 2012). In crossbred cows, treatment with α-tocopherol acetate during dry period resulted in reduced oxidative stress and HSP70 (Aggarwal et al. 2013). In cultured rat hepatocytes vitamin E significantly counteracted the effect of cyclosporine A-induced increase in HSP70 (Andrés et al. 2000). However, in young men, γ-tocopherol was shown to prevent the exercise-induced increase of HSP72 in skeletal muscle as well as in the circulation (Fischer et al. 2006).

However, in most of cases effect of vitamin E on HO-1 expression is different from the aforementioned effects on HSP70. Indeed, recently it has been shown that vitamin E activated the HO-1 promoter via the cAMP-response element but not the ARE enhancer through the extracellular signal-regulated kinase and protein kinase A (Reed et al. 2015). It was shown that α-Tocopheryl succinate (α-TOS) increases nuclear translocation and electrophile-responsive/antioxidant-responsive elements binding activity of Nrf2, resulting in up-regulation of downstream genes cystine-glutamic acid exchange transporter and HO-1, while decreasing NF-κB nuclear translocation (Bellezza et al. 2012). It seems likely that α-tocopherol protects human retinal pigment epithelial cells from acrolein-induced cellular toxicity, not only as a chain-breaking antioxidant, but also as a Phase II enzyme inducer, including Nrf2, SOD and HO-1 induction (Feng et al. 2010). Similarly, in a murine prostate cancer model γ-tocopherol-enriched mixed tocopherols significantly upregulated the expression of Nrf2 and its related detoxifying and antioxidant enzymes, including SOD and HO-1 (Barve et al. 2009). In rats, protective effect of vitamin E against focal brain ischemia and neuronal death was shown. In fact, vitamin E induced the expression of the alpha subunit of hypoxia-inducible factor-1 (HIF-1) and its target genes, including vascular endothelial growth factor (VEGF) and heme oxygenase-1 (Zhang et al. 2004).

5.1.3.4.2 Ascorbic Acid

Ascorbic acid is main water-soluble antioxidant provided with feed and synthesised within the animal/chicken body (Chakraborthy et al. 2014). It has been shown that chickens experience a less severe stress response after exposure to high temperatures when they are provided dietary ascorbic acid. In fact, heart HSP70 expression decreased in ascorbic acid-fed chickens and the HSP70 increase after heat was two-fold lower in ascorbic acid-fed birds in comparison with the control chickens. Furthermore, plasma corticosterone and heart HSP70 were positively correlated, while plasma ascorbic acid and heart HSP70 were negatively correlated (Mahmoud et al. 2004b). In the ascorbic acid-fed chickens, neither the lower constitutive HSC70 nor the decreased HSP70 response to heat stress (42 °C) in the heart and liver were sex-dependent (Mahmoud et al. 2003). A lower expression of HSP70 associated with lower body temperature in heat-stress conditions reflected a lower stress response in the ascorbic acid-fed birds. Indeed, ovary and brain HSP70 expression linearly decreased as dietary vitamin C or vitamin E supplementation increased in heat-stressed quail. However, HSP70 expression of ovary and brain was not affected by vitamin C or E supplementation under thermo-neutral conditions (Sahin et al. 2009).

Effects of ascorbic acid on HSP70 expression were also evaluated in experiments with laboratory animals or in human trials. For example, lymphocytes from non-supplemented subjects responded to hydrogen peroxide with increased HSP60 and HSP70 content over 48 h. In fact, in vitamin C supplemented subjects, baseline HSP60 (lymphocytes) and HSP70 (muscles) content were elevated, but they did not respond to hydrogen peroxide or exercise (Khassaf et al. 2003). In elderly, increased concentration of vitamins C and E was associated with a reduction in oxidative stress and leukocytes HSP72 (Simar et al. 2012). Ascorbic acid was shown to attenuate increase in HSP expression due to various toxic agents or heat stress. For example, human brain astrocyte cells enriched with ascorbic acid before being exposed to ethanol, were reported to be better protected against the alcohol-mediated toxicity than non-supplemented cells, and showed significantly lower concentrations of HSP70 9 (Sánchez-Moreno et al. 2003). Ascorbic acid significantly attenuated Cd-induced upregulation of GRP78 in mouse testes (Ji et al. 2012). Cyclic heat stress (23 to 38 to 23 °C, for 2 h on each of seven consecutive days) activated hepatic HSP70, TNF-α, iNOS, and GSH-Px genes, whereas vitamin C (0.5% in water) during heat stress ameliorated heat stress-induced cellular responses in rats (Yun et al. 2012). It is interesting to note that there was a specific disappearance of HSP70 in the testes of 20-day-old ascorbic acid-deficient mice (Yazama et al. 2006). It seems likely that effects of ascorbic acid on HSP is not universal and for HO-1 is different from HSP70. Indeed, the HO-1 mRNA and protein level in rat kidney, liver, and lung were highly induced by ascorbate treatment (100 mg/kg b.w.) under normal and HS conditions. In particular, in HS the HO-1 activity in tissues was enhanced by both ascorbate pre- and post-treatment (Zhao et al. 2014).

5.1.3.4.3 Vitamin D3

Vitamin D is known for its classical functions in calcium uptake and bone metabolism. However, recently, vitamin D has been recognized for its non-classical actions including modulation of antioxidant defenses (Zhong et al. 2014; Xu et al. 2015) through regulating oxidant and antioxidant enzyme genes. It was shown that HO-1 was down-regulated in the livers of mice fed the vitamin D deficient diet (Zhu et al. 2015). At the same time, vitamin D deficiency increases the expression of the hepatic mRNA levels of HO-1 in obese rats (Roth et al. 2012). In a model of reperfusion of bilateral femoral vessels pre-treatment of rats with vitamin D3 results in a significant increase in leukocyte HO-1 expression in rat model of reperfusion (Shih et al. 2011). By employing microarray technology, the effect of a single dose of 1,25-(OH)2D3 on gene expression in the intestine of vitamin D-deficient rats was assessed. Indeed, at 3 h, there was a 1.9-fold increased expression of HO-1 (Kutuzova and DeLuca 2007). The effects of 1,25-D3 treatment on HO-1 expression following focal cortical ischemia elicited by photothrombosis in glial cells were studied. Postlesional treatment with 1,25-D3 (4 μg/kg body weight) resulted in a transient, but significant upregulation of glial HO-1 immunoreactivity (Oermann et al. 2004).

5.1.3.4.4 Carnitine and Betaine

Carnitine is considered as a novel mitochondria-targeted antioxidant with a range of antioxidant actions (Surai 2015a), while betaine is reported to have antioxidant properties in various oxidative stress-generating model systems (Alirezaei et al. 2015). In human endothelial cells in culture carnitine was shown to increase gene and protein expression of HO-1 (Calò et al. 2006). Furthermore, in humans and in an animal model it was shown that carnitine-mediated improved response to erythropoietin involves induction of HO-1 (Calò et al. 2008). Indeed, L-carnitine treatment was associated with an increased level of HO-1 in the retinal ganglion cells (Cao et al. 2015). L-Carnitine prevented increase in HSP70 in the testes of cadmium-exposed rats (Selim et al. 2012). It was shown that Acetyl-L-carnitine-induced up-regulation of heat shock proteins protects cortical neurons against amyloid-beta peptide 1–42-mediated oxidative stress and neurotoxicity (Abdul et al. 2006). Acetylcarnitine induces heme oxygenase (increased the amount and activity of HO) in rat astrocytes and protects against oxidative stress (Calabrese et al. 2005). From the aforementioned data it is clear that carnitine can be considered as an important regulator of the vitagene network.

The influence of hyperosmotic shrinkage and the osmolyte betaine on heme oxygenase HO-1 expression was studied in cultured rat hepatocytes. Hyperosmolarity transiently suppressed HO-1 induction in response to hemin or medium addition at the levels of mRNA and protein expression. Pretreatment of the cells with betaine largely restored induction of both HO-1 mRNA and protein under hyperosmotic conditions (Lordnejad et al. 2001).

5.1.3.4.5 Selenium

Selenium is a central part of the antioxidant defence network via at least 25 selenoproteins (Surai 2006). The protective effect of selenium against cadmium-induced cytotoxicity in chicken splenic lymphocytes was shown to be mediated via the HSP pathway (Chen et al. 2012). Indeed, the mRNA expression of HSP (HSP27, HSP40, HSP60, HSP70 and HSP90) exposed to 10^{-6} mol/L Cd showed a sustained decrease at 12–48 h exposure. In contrast, adding to the medium Se (10^{-7} mol/L) was associated with a significant increase in the mRNA expression of HSP, as compared to the control group of chicken splenic lymphocytes. Concomitantly, treatment of chicken splenic lymphocytes with Se in combination with Cd prevented a decrease in the mRNA expression of HSP due to Cd treatment. A different HSP response to arsenic was observed. The expression of HSP mRNA and protein (HSP70 and HO1) in rat liver were increased by 5 and 3 folds in the arsenic-fed animals compared with the control group, and selenium prevented the occurring of oxidative damage from arsenic and significantly reduced expression of HSP mRNA and protein (Xu et al. 2013).

The HSP70 response was shown to be significantly lower in the chickens fed selenium and challenged with either enteropathogenic Escherichia coli or heat stress than in those chickens given no supplemental selenium (Mahmoud and Edens 2005; 2003). An acute heat stress induced HSP70 in 22d turkey embryos and the embryos from selenium-fed dams were shown to have less HSP70 after the 3 h post-heat stress recovery period (Rivera et al. 2005) demonstrating that selenium had the ability to reduce the impact of heat stress. In fact, heat stress enhanced HSP70 and HSP27 expression and concentration in chicken spleen and dietary Se prevented the aforementioned increase in HSP (Xu et al. 2014). Similarly, in piglets under heat stress conditions selenium can down-regulate the mRNA levels of HSP in various tissues (Gan et al. 2013a). The relative messenger RNA (mRNA) and protein expression of HSP60, HSP70, and HSP90 in PBMC was observed highest in heat-stressed goats and Se + vitamin E supplementation decreased the HSP expression (Dangi et al. 2015).

In contrast, Se deficiency increased the mRNA levels of HSP (HSP90, 70, 60, 40, and 27) in chicken neutrophils (Chen et al. 2014). Indeed, HSP played an important role in the protection of the chicken liver after oxidative stress due to Se deficiency. For example, the mRNA levels of HSP and the protein expression of HSP (HSP60, 70, and 90) increased significantly in the Se-deficient group compare to the corresponding control group (Liu et al. 2015a). In exudative diathesis (ED) broiler chicken model caused by Se deficiency, the antioxidant function was shown to decline remarkably, and most of the HSP expression levels increased significantly in the spleen, thymus, and bursa of Fabricius of the broiler chicks with ED (Yang et al. 2016). Se deficiency causes defects in the chicken bursa of Fabricius associated with decreased selenoprotein expression (Khoso et al. 2015). As a compensatory response to changes due to Se deficiency, the mRNA and protein expression levels of HSP (HSP27, HSP40, HSP60, HSP70, and HSP90) were significantly increased. Similar observations with Se deficient mouse were

recorded. For example, Se deficiency was shown to increase HSP70 levels in mouse testis (Kaur and Bansal 2003). A significant increase in the stress-inducible HSP70 gene and protein expression was observed in the mice fed Se-deficient or Se-excess supplemented diet as compared with Se adequate fed group (Kaushal and Bansal 2009). It is interesting to note that the testis-specific HSP70–2 expression significantly decreased as result of Se deficiency. It is clear that increased expression of HSP in response to toxic metals is an adaptive mechanism to deal with oxidative stress imposed by such toxicants. Similarly, in the case of Se deficiency increased HSP expression is also an adaptive mechanism to compensate for lack of synthesis of selenoproteins and their antioxidant protective functions

As mentioned above, HSP response to various stressors and to nutritional supplements would depend on many factors, including the model used, stressor nature and strength, etc. For example, in human lens epithelial cells sodium selenite gradually increased the expression of HSP70 in a time-dependent manner (Zhu et al. 2011). In rat hippocampus with ischemia-induced neuronal damage, selenium pretreatment was shown to significantly increase the level of HSP70 when compared with ischemic group (Yousuf et al. 2007). In fact, a significant increase in hippocampal HSP70 expression in the ischemic group was observed but the expression was even higher in the selenium-pretreated group than ischemic group.

5.1.3.4.6 Phytochemicals

Regulatory and health promoting properties of various phytochemicals and their effects on HSP have received substantial attention and there is a range of comprehensive reviews covering the subject (Mattson and Cheng 2006; Calabrese et al. 2011; Murakami 2013; de Roos and Duthie 2015). They are beyond the scope of the present review. Therefore, only effects of silymarin, possessing a range of antioxidant-related activities (Surai 2015b), are reviewed below.

5.1.4 Silymarin

It seems likely that SM, similar to other flavonoids, can affect the vitagene network. In fact, SM/silybin affects HSP32 (HO-1) activity in different model systems. For example, As-intoxicated rats showed a significant up-regulation of myocardial NADPH (NOX) oxidase sub-units such as NOX2 and NOX4 as well as Keap1 and down-regulation of Nrf2 and vitagene HO-1 protein expressions. Pre-administration of silibinin (75 mg/kg/BW) recovered all these altered parameters to near normalcy in As-induced cardiotoxic rat (Muthumani and Prabu 2014). Similarly, in a model of liver injury caused by alcohol plus pyrazole, SM administration (50 mg/kg/BW) had a protective effect with a trend in restoring the decreased activity of HO-1 and Nrf2 (Choi et al. 2013). SM (250 mg/kg/BW) possesses substantial protective effect against B(a)P-induced damages by increasing (restoring) HO-1 (vitagene) activity

(Kiruthiga et al. 2015). Similarly, in vitro SM (500 μM) reduced tBH-induced hepatocyte toxicity by activating HO-1 gene expression (Cerný et al. 2009). Indeed, the enzyme HO-1 is an important regulatory molecule present in most mammalian cells. In fact, the main function of HO-1 is to break down the pro-oxidant molecule heme into three products; carbon monoxide (CO), biliverdin and free iron and actively participate in the antioxidant defence in the human/animal body (Venditti and Smith 2014). Indeed, HO-1 is a stress-inducible protein and can be induced by various oxidative and inflammatory signals. From the data presented above it is clear that SM/silibinin can upregulate HO-1 and improve antioxidant defences. It is likely that SM/silibinin can affect other HSP including HSP70. Indeed, in an in vitro system based on CHO-K1 cells treated with As, SM (5 μM) significantly decreased HSP70 expression previously elevated by As (Bongiovanni et al. 2007). In another in vitro system based on heat-induced chicken hepatocytes, SM (259 μM) affected HSP70 expression significantly, preventing its alleviation by heat stress (Oskoueian et al. 2014). A similar protective effect of SM (100 mg/kg/BW) on HSP70 was seen in rats given SM for 7 days prior to mesenteric ischemia-reperfusion (I-R) compared to I-R group (Demir et al. 2014). It is interesting to note that silybin was identified as a novel HSP90 inhibitor (Zhao et al. 2011). Therefore, silibinin can decrease HSP70 expression in stressed cells indicating improved AO defences and decrease stress by other means (e.g., Nrf2-related increased AO synthesis). Indeed, effects of silymarin on HSP in avian species awaits investigation, while other phytochemicals are shown to be effective. For example, resveratrol, a plant phytochemical possessing antioxidant activities, attenuated the heat stress-induced overexpression of HSP27, HSP70, and HSP90 mRNA in the bursa of Fabricius and spleen and increased the low expression of HSP27 and HSP90 mRNA in thymus in 42 d old chickens upon heat stress (Liu et al. 2014). Indeed, there is a need for more detailed investigation of the relationship between nutritional antioxidants and HSP expressions in physiological and stress conditions.

5.1.5 Nutritional Modulation of Vitagenes

The aforementioned data clearly indicate that vitagenes can be modulated by nutritional means. Indeed, vitamins E, D, C, carnitine, betaine, selenium and some phytochemicals can affect HSP expression and concentration in various stress conditions. It is interesting that the same compounds can affect other vitagenes, namely thioredoxins, sirtuins and SOD (Surai and Fisinin 2012). Therefore, it would be of considerable interest to develop an antioxidant-based composition/supplement for decreasing negative consequences of various stresses in poultry and pig production. Such a composition should meet at least five important requirements (Surai 2015a):

1. Vitagene activation and redox-signaling (carnitine, betaine, vitamins A, E, D, C, Se, Zn, Mn, silymarin and possibly other phytochemicals);

2. Maintenance of the vitamin E recycling system (vitamin C, Se, Vitamin B1 and B2);
3. Provision of nutrients required for carnitine synthesis (lysine and methionine, ascorbic acid, vitamin B6 and niacin);
4. Supporting the liver, a main site of T-2 toxin, ochratoxins, fumonisins and aflatoxins detoxification and gut, responsible to DON detoxification (vitamins E and C, selenium, carnitine, betaine, organic acids, methionine and lysine);
5. Possessing immunomodulating properties (vitamins A, E, D, C, carnitine, Se, Zn and Mn).

Inclusion of various protective compounds into the diet of farm animals and poultry to decrease negative consequences of stress conditions is complicated, firstly, by a decreased feed consumption at time of stress. Secondly, such an approach has a low flexibility, since existing feeding systems do not allow to include anything into the feed loaded into the feed storage bins located near the poultry/pig house (usually several tons of feed for several days feeding). Therefore, before the previous feed is consumed, nothing can be added to the feed. However, sometimes it is necessary to supplement animals/poultry with specific additives very quickly to deal with consequences of unexpected stresses (e.g. mycotoxins in the feed, immunosuppression, high temperature, etc.). In such a case, additive supplementation via drinking system is a valuable option (Surai and Fisinin 2012). In fact, modern commercial poultry and pig houses have water medication equipment installed, which can be perfectly used for the aforementioned supplementations. For example, an attempt to address the aforementioned option was implemented in a commercial product PerforMax, containing a vitagene-regulating mixture of 28 compounds, including antioxidants (vitamins E and C), carnitine, betaine, minerals (Zn and Mn) and essential amino acids, and supplied via drinking water. Its efficacy in fighting stresses in commercial poultry production has been recently reviewed (Shatskih et al. 2015) and prospects of its use to maintain gut health in weaned piglets and newly hatched chicks was considered (Surai and Fisinin 2015). Indeed, it is well known that commercial animal/poultry production is associated with a range of stress conditions including environmental (high temperature), nutritional (mycotoxins and oxidized fat) or internal (vaccinations, disease challenges, etc.) stresses (Shatskih et al. 2015; Surai 2002, 2006). In such conditions, supplying the PerforMax with drinking water was shown to have protective effects in growing birds (Fotina et al. 2014; Velichko and Surai 2014) as well as in adult birds (Shatskih et al. 2015) helping maintain their health, productive and reproductive performance. Therefore, the aforementioned results are the first step to go from the development of the vitagene concept to designing a commercial product and testing it in the commercial conditions of poultry and pig production. We can suggest that this idea could be realized in human nutrition as well. Clearly more research is needed to understand a fundamental role of vitagenes in adaptation to various stresses.

5.1.6 Conclusions and Future Directions

From the aforementioned analysis of the data related to HSP in poultry physiology and adaptation to stresses it is possible to conclude:

- HSP as important vitagenes are main driving force in cell/body adaptation to various stress conditions. Indeed, in stress conditions synthesis of most cellular proteins decreases while HSP expression is usually significantly increased.
- HSP being cellular chaperones are responsible for proteostasis and involved in protein quality control in the cell to prevent misfolding or to facilitate degradation, making sure that proteins are in optimal structure for their biological activities
- There are tissue-specific differences in HSP expression which also depends on the strength of such stress-factors as heat, heavy metals, mycotoxins and other toxicants.
- HSP70, HSP90 and HSP32 are shown to be protective in heat stress, toxicity stress as well as in other oxidative-stress related conditions in poultry production
- Molecular mechanisms of HSP participation in acquisition of thermotolerance need further detailed investigation
- There are complex interactions inside the antioxidant systems of the cell/body to ensure an effective maintenance of homeostasis in stress conditions. Indeed, in many cases nutritional antioxidants (vitamin E, ascorbic acid, selenium) in the feed can decrease oxidative stress and as a result HSP expression could be decreased as well
- Regulating effects of various phytochemicals on HSP need further investigation
- Protective effects of HSP in immunity in stress conditions await practical applications in poultry production
- Nutritional means of additional HSP upregulation in stress conditions of poultry production and physiological and commercial consequences await investigation. Indeed, in medical sciences manipulation of HSP expression is considered as an important approach in disease prevention and treatment. It seems likely that in poultry/animal sciences nutritional manipulation of vitagenes is a new way in managing commercially-relevant stresses.

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Chapter 6
Heat Shock Protein and Thermal Stress in Chicken

Shanmugam Murugesan, Rajkumar Ullengala, and Vinoth Amirthalingam

Abstract Chicken has been selected for higher production performance over the years and are highly sensitive to changes in their environment. The average global temperature has increased over the century and is further expected to rise. In open house rearing system chicken is vulnerable to this increasing environmental temperature and may experience thermal stress. Heat shock proteins (HSP) are highly conserved family of proteins playing important role in normal cellular physiology and cytoprotection against different stressors including heat stress. In chicken levels of different members of HSP family are increased in almost all the tissues in response to heat stress. This increased HSP level protects cellular proteins from heat stress induced damage. Efforts to overcome the heat stress conditions in chicken have lead to development of thermal manipulation protocols whereby epigenetic modifications are introduced. Through epigenetic adaptation the birds acquire protection against the adverse effects of heat stress. This chapter discusses the findings on cellular HSP responses to heat stress and the thermal manipulation strategy to overcome heat stress in chicken.

Keywords Chicken · Epigenetics · Heat stress · Heat shock proteins · Hsp70 · Thermal manipulation

Abbreviations

CO_2	Carbon dioxide
HSP	Heat shock protein
RH	Relative humidity
TM	Thermal manipulation

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6.1 Introduction

The global mean surface temperature has increased by 0.85 °C over the period (1880–2012) and by the end of this century it is further expected to increase 0.3 to 4.8 °C under different scenarios (IPCC 2014). The optimum temperature range for production for broiler and layer chicken is 18 to 22 °C (Charles 2002; Aengwanich and Simaraks 2004) with slight variations on either side due to differences in age, sex and line/strain. In the tropics and sub tropics chicken are generally reared in open-sided houses. This makes chicken more vulnerable to high environmental temperature during summer and is likely to experience heat stress. This is a challenging situation for the commercial poultry industry in terms of sustaining production and animal welfare.

Chicken are homeothermic and the body temperature is maintained in the range of 41 to 42 °C. Chicken are more sensitive to high ambient temperatures due to lack of sweat glands and high body temperature. Furthermore, the fast growing commercial broilers and layers producing high number of eggs generate metabolic heat that adds to the already stressful situation during summer. Birds selected for fast growth exhibit a reduced thermoregulatory capacity in comparison to layers and are therefore more susceptible to heat stress and related problems (Mitchell et al. 2005). During heat stress evaporative cooling is an important mechanism for body temperature control. In birds evaporative cooling involves evaporative mechanisms involving skin and respiratory system. The gradient between body surface and air moisture determines the rate of evaporative cooling. When high environmental temperature combined with high relative humidity prevails the evaporative cooling mechanism is impeded. Thus the combined effects of high ambient temperature with high humidity results in hyperthermia leading to decreased food consumption, growth rate, feed efficiency, total egg production, egg quality and survivability (Mashaly et al. 2004; Rajkumar et al. 2011; Xie et al. 2014). The performance of birds gets affected when the ambient temperature increases and approaches the upper critical temperature and above this point the body temperature increases until it reaches the lethal stage. Under heat stress conditions birds try to adapt themselves by increasing peripheral blood flow that helps in dissipating excess body heat, decreases feed intake so as to reduce heat increment and increases panting to facilitate evaporative cooling (Daghir 2008). A variety of cellular structures and metabolic processes are damaged by heat stress and the extent of damage is based on magnitude and duration of heat exposure.

All organisms respond to heat stress by inducing a set of proteins called as Heat Shock Proteins (HSP). The HSP are synthesized in cells in response to different types of stressors. Ferruccio Ritossa (1962) reported chromosomal puffing pattern and higher transcriptional activity in Drosophila salivary glands after exposure to heat. Later the transcriptional activity was discovered to be synthesis of heat induced polypeptides and named as heat shock proteins (Ashburner and Bonner 1979). HSP are highly conserved proteins consisting of several families found in all living organisms (Lindquist and Craig 1988). The HSP act as molecular chaperons that

help in protein folding and assembly, assist in restoring the native state of protein, regulate degradation of protein and in translocation across membranes (Hartl and Hayer-Hartl 2002). HSP synthesis up-regulation under different stress conditions is an adaptive phenomenon resulting in improved tolerance. There exists a relationship between thermo tolerance and HSP synthesis in organisms (Parsell and Lindquist 1994).

6.2 Heat Stress

Lee (1965) has defined stress as "the magnitude of forces external to the bodily system which tend to displace that system from its resting or ground state". Stress may be caused by many factors including climate in which birds are reared. The ambient temperature range within which birds do not use additional energy to maintain the body temperature is known as thermoneutral zone. Weather conditions above this thermoneutral zone for prolonged time results in heat stress. Heat stress is a result of interaction between ambient temperature and relative humidity and in birds this interaction effect is complex (Yahav et al. 1995). In birds, excess body heat is dissipated through conduction, convection, radiation and water evaporation through respiratory tract. The physiological events that occur during the period of heat stress are described in Fig. 6.1. Among different heat loss mechanisms blood

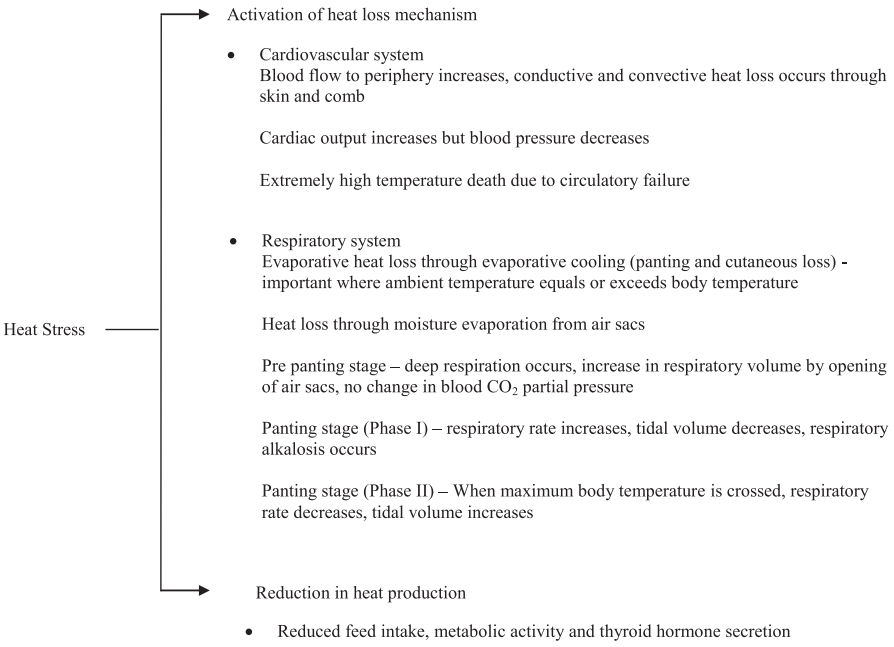

Fig. 6.1 Physiological response to heat stress

flow redistribution and passive water loss from respiratory system are important. Water loss during prolonged heat stress condition results in dehydration and decreased blood volume. This decreased blood volume alters the venous return and blood circulation to upper respiratory tract culminating in hyperthermia and finally death. The physiological changes due to heat stress are accompanied by damaging changes at cellular level. There is significant increase in induction of different members of heat shock protein family during heat stress. The different events occurring at cellular level during heat stress are:

- Increase in membrane liquidity
- Destabilization of membrane components
- Cytoskeleton modification
- Mitochondrial swelling
- Endoplasmic reticulum and golgi apparatus fragmentation
- Nucleolus disintegration
- Formation of electron dense granules – stress bodies – in nucleus
- Decrease in cell viability and cell death (depending on heat stress intensity, type of cells and stage of cell cycle)(Velichko et al. 2013)

Birds respond differently to acute and chronic heat stress. Plasma metabolites are primarily disturbed in acute heat stress and tissue damage occurs in chronic heat stress (Xie et al. 2015). Acute heat stress results in increase in body temperature and increase in respiration rate to 235 breaths/min from 21.7 breaths/min observed during thermoneutral conditions (Mitchell and Siegel 1973). The capillary blood flow to exterior of body increases whereas to the internal organs it decreases (Wolfenson et al. 1981). Though heat stress affects the production in both layers and broilers the effect is more pronounced in commercial broilers and broiler breeders because of their heavy body. The huge muscle mass of broilers posses challenge in excess heat dissipation during heat stress conditions resulting in higher core body temperature and fatality. The magnitude of heat tolerance is genetically variable and related to the growth potential of the bird (Settar et al. 1999).

6.2.1 Heat Shock Proteins

Heat shock proteins are expressed in cells constitutively or induced by different stimuli such as heat, chemical etc. HSP are classified into six families based on molecular weights namely HSP100, HSP90, HSP70, HSP40, small HSP and chaperonins (De Maio and Vazquez 2013). Apart from the molecular chaperone activity small HSP are also implicated in other cellular functions such as stress tolerance, protein folding, cytoskeletal integrity and cell cycle (Bakthisaran et al. 2015). HSP are also found in the extracellular environment, secreted actively or passively, where they may act as stress signals and stimulates immune cells (De Maio and Vazquez 2013). HSP90 is the most abundant soluble protein comprising 1–2% of total cell proteins and under stress conditions it is upregulated (Csermely et al. 1998). The

cytoplasmic HSP90 is found in two forms namely inducible HSP90α and constitutive HSP90β. In mammalian cells HSP27 expression has been shown to be correlated with thermotolerance (Landry et al. 1989).

6.2.2 Heat Stress and HSP

Natural incubation conditions may not be uniform as hens regularly move in search of food and there may be non-uniform nest insulation. Heat stress may affect the developing embryos and induct HSP during natural incubation. Reports indicate HSP induction in chick embryos after exposure to high incubation temperature for various durations. Chicken embryo fibroblasts exposed to 45 °C was observed to increase the synthesis of three proteins (MW 22,000, 76,000 and 95,000 daltons), the level was exceeding the synthesis of cell structural proteins. The level of these proteins returned back to normal after 8 h (Kelley and Schlesinger 1978). High temperature increasing some HSP expression in chicken embryonic cells was reported by Edington and Hightower (1990). Exposing chicken embryonic cells to 44 °C for 30 minutes caused two to three fold increases in HSP23, HSP71 and HSP88 proteins expression. Similarly, exposing developing chick embryos to higher incubation temperature than normal increased different HSP gene and protein expressions (Gabriel et al. 2002; Vinoth et al. 2015).

The testes in birds are located inside the body with an internal temperature range of 40–41.7 °C and spermatogenesis is efficient even at this temperature. This is because of HSPA2 gene in bird testes that provides an adaptive advantage over the mammals (Padhi et al. 2016). However, the internal location of testes also predisposes it to heat stress induced hyperthermia that affects spermatogenic process. The impact of high ambient temperature on semen quality and fertility is severe in broiler males having best semen quality index (Karaca et al. 2002). Acute heat stress (38 °C and 55% RH for 4 h) in Taiwanese roosters caused upregulation of genes belonging to HSP family (HSP70, HSP90AA1, HSPB8, HSPA5, HSPA8, HSPB1, HSPA4) in the testes (Wang et al. 2013). However, in a subsequent report it was shown that acute heat stressed rooster testes had decreased expression of HSP90α and HSP25 proteins that are involved in protein folding (Wang et al. 2014). Besides the action of preventing aggregation of misfolded proteins and protein repair HSP70 has important role in spermatogenesis. The molecular chaperone HSPA2 is a member of the HSP70 family. It has also been shown to play important role in spermatogenesis and male fertility. In response to heat stress HSP25, HSPD1, HSPA5 and HSPA2 gene expression in chicken testes is upregulated (Wang et al. 2015). The genes HSPD1 and HSPA5 are involved in apoptotic process. Thus higher expression of these two genes indicates occurrence of apoptosis in testicular cells. There is difference in gene expression of the HSPD1 in testis of heat stressed broiler and layer type chicken (Wang et al. 2015). Chicken testes subjected to heat shock (46 °C for 2 h) had increased expression and polyadenylation of HSP70 and was suggested

that this might contribute to development of thermotolerance (Mezquita et al. 1998). HSP70 is expressed constitutively in chicken testes and the expression increased when subjected to heat shock at 44 °C or 46 °C (Mezquita et al. 2001). Taiwanese country chicken subjected to heat stress conditions for six weeks had higher sperm HSP70 concentration, however, semen samples kept at 41 °C had decreased or no change in HSP70 concentration depending on the semen diluent used (Yan 2001). The differing result of HSP70 in this experiment may be due to difference between in vitro and in vivo conditions. During heat stress the increase in HSP expression is an indirect indication of disturbance in testes physiology and consequent heat stress related fertility problems.

Exposure of chicken to acute heat stress of 40 °C for duration spanning 1 and 1.5 h resulted in higher HSP70 gene expression in brain. There was no difference in HSP70 gene expression between native chickens but the commercial layer chicken had higher expression (Tamzil et al. 2013). The susceptibility of commercial layers to acute heat stress might be due to higher production performance. Furthermore, HSP70 gene expression during heat stress was influenced by HSP70 genotype of the chicken. Chicken having certain HSP70 genotype was found to be heat tolerant and acute heat stress had no negative effect on growth performance and egg production (Liang et al. 2016).

Heat stress causes variety of changes in broiler gastrointestinal tract such as alteration in intestinal microflora, intestinal injury and intestinal barrier integrity impairment (Quinteiro-Filho et al. 2010; Song et al. 2014). In the process of heat dissipation blood flow is diverted to the periphery of the body and the functions of internal organs such as intestine get adversely affected. Furthermore, the susceptibility to heat stress differs with the region of the intestine. Broilers subjected to heat stress had upregulated expression of HSP70 and HSP90 mRNA expression in jejunum and ileum, however, the response was more pronounced in ileum than in jejunum. The HSP mRNA expression was unaltered in duodenum and colon (Varasteh et al. 2015). At the protein level only HSP70 and not HSP90 was increased in jejunum and ileum.

Hypothalamus is crucial for detection and regulation of body temperature. Taiwan broiler type country chicken subjected to acute heat stress (38 °C for 2 h) had upregulated HSPB1 and HSP25 through the recovery period indicating their essential role in the hypothalamus (Tu et al. 2016). HSP70 gene expression increased but expression of HSP90α and HSP90β were unaltered in brain of heat stressed chicken (Mahmoud et al. 2004; Zhen et al. 2006; Zhang et al. 2014). Furthermore, this induced expression of HSP70 mRNA in brain differed with the HSP70 genotype of the chicken (Zhen et al. 2006). HSP70 protein expression increases in brain and liver of heat stressed but not in heat acclimated broilers (Guerreiro et al. 2004).

Liver is highly metabolically active organ and therefore, comparatively is more susceptible to heat stress than other organs. Acute heat stress increased HSP90α, HSP90β, HSP70 and HSP60 expressions in broiler liver and HSP60 expression gradually decreased at 3 h of exposure (Gabriel et al. 1996; Mahmoud et al. 2004; Zhen et al. 2006; Yu and Bao 2008; Yu and Bao 2009; Yan et al. 2009; Zhang et al. 2014). Broilers subjected to chronic heat stress for 3 or 6 weeks had higher rectal

temperature and up-regulated liver HSP70 mRNA expression (Givisiez et al. 1999; Zuo et al. 2015). RNA-seq technology was applied to study the liver transcriptome response to acute and chronic heat stress and found that broilers differentially expressed more genes than heat resistant Fayoumi line (Lan et al. 2016). Gene expression response to acute heat stress was stronger compared to chronic heat stress in broilers. Furthermore, this response differed between the chicken lines studied. Network analysis of differentially expressed genes indicated HSP having numerous connections with immune related genes. Furthermore, the secreted HSP tend to attract immune cells (Shevtsov and Multhoff 2016). In another study using RNA seq method, chicken hepatocarcinoma cell line maintained at 43 °C for 2.5 h had several HSP upregulated with HSP25 having the highest expression (Sun et al. 2015). Liver of laying broiler breeder had increased HSP70 expression during acute heat stress and HSP90 expression during chronic heat stress (Xie et al. 2014).

HSP70 and HSP90 gene expression in muscle were higher during acute heat stress but not in chronic heat stress (Zhen et al. 2006; Xie et al. 2014; Zhang et al. 2014). Similarly acute heat stress caused increase in HSP90 and HSP70 protein and mRNA expression in kidney of broilers (Yu and Bao 2008; Yu and Bao 2009).

HSP60, HSP70 and HSP90 mRNA expressions were increased in broiler heart after 2 h of heat exposure and subsequently expression declined except for HSP90 which expression again increased at 10 h of heat exposure. However, the corresponding proteins were increased in concentration and remained till 10 h post exposure (Yu and Bao 2008; Yu et al. 2008; Yu and Bao 2009) indicating a prolonged adverse effect of heat exposure. One hour exposure of broilers to high environmental temperature (40/41 °C) caused increase in expressions of HSP90α, HSP90β and HSP70 in heart (Mahmoud et al. 2004; Zhang et al. 2014). Apart from heart, HSP90 and HSP70 proteins were detected in the broiler heart blood vessels by immunolocalization (Yu et al. 2008; Yu and Bao 2009). In another study, during chronic heat stress HSP70 mRNA expression was upregulated and during acute heat stress HSP90 mRNA expression upregulated in heart of laying broiler breeders (Xie et al. 2014). The difference in the expression pattern among the reports may be due to the age difference of the birds used in the experiments. In vitro and in vivo high temperature exposure induced higher levels of HSP 23, 70 and 90 expressions in broiler chickens peripheral leukocytes (Wang and Edens 1998). Layer chicken subjected to long term moderate heat stress had higher HSP70 protein concentration in circulating mononuclear blood cells but the response to heat stress was not uniform at different ages studied (Maak et al. 2003). Exposing chicken macrophage-line HD11 cells to 45 °C for 2 h caused increase in mRNA expression of chaperone genes HSP25, HSPA2 and HSPH1 (Slawinska et al. 2016). The manifold expressed small HSP (HSP25) is ATP independent chaperone that bind to unfolded proteins and other HSP engage with these proteins to bring back to their original structure. Acute heat stress increases HSP90α, HSP90β and HSP70 expression in broiler spleen (Mahmoud et al. 2004). The body temperature of heat stressed young White Leghorn chickens were positively correlated with HSP70 expression in heart and liver (Mahmoud et al. 2003). High ambient temperature severely affects egg production and quality (Rozenboim et al. 2007). Apart from reduced feed intake and altered

physiological parameters of the hen (Darre and Harrison 1987) change in gene expressions of small ovarian follicles in the laying hens was reported. HSP25 gene expression was upregulated indicating damage to proteins of follicle cells (Cheng et al. 2015). There is 70–80% fall in capillary blood flow to large ovarian follicles and infundibulum in heat stressed laying hens (Wolfenson et al. 1981). Collectively, low blood flow and ovarian follicle cellular damage may be the reasons for reduction in egg production in laying hens.

The mRNA expression of HSP70 in tissues during heat stress differs depending on the breed of chicken, some were more tolerant to high ambient temperature than others (Zhang et al. 2014). Even the basal expression of HSP70 mRNA in liver and muscle differs with the HSP70 genotype of the chicken, expression being higher in heterozygous genotypes than that of homozygous genotypes (Zhen et al. 2006). A global gene expression study using microarray in acute heat exposed broiler chicken revealed differentially expressed genes including HSPH1 and HSP25 in brain, liver and leg muscle (Luo et al. 2014). These HSP genes were reported to be part of different functional clusters that were related to the effects of heat stress.

A clear difference in HSP gene expressions was found between the tissues. This might be due to the functional or metabolic status of the tissues. Likewise a difference in HSP gene expression was found between the chicken lines. The duration of heat exposure, acute or chronic, differentially affected HSP gene expressions. HSP are expressed in almost all vital organs studied in response to heat stress in chicken. The functional relationship between the HSP expression and other systems/process vis-à-vis production in chicken will be helpful to address the production and welfare of chicken during heat stress conditions.

6.3 Epigenetics and Thermal Manipulation

The term epigenetics was introduced by Conrad Waddington (1942) and defined it as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being.” However, at present there is lack in clear definition of the term (Deans and Maggert 2015). It is now accepted that apart from information contained in DNA sequence the overall phenotype is also determined by epigenetic information. The epigenome is generated by different epigenetic processes such as DNA methylation, chromatin remodeling, post translational histone modifications, regulation of gene expression by non-coding RNA’s instability of genome and modification of animal phenotype by other forces (Triantaphyllopoulos et al. 2016). Epidemiological studies in humans and genetic studies in animals have revealed that epigenetic marks, in addition to the DNA sequence, may be transmitted across generations and influence the offspring phenotype (Jablonka 2009; Nätt et al. 2012).

Body temperature regulation system, similar to most functional systems, during embryogenesis develops from open loop control systems into closed control systems (Dörner 1974). The preoptic anterior hypothalamic area contains thermosensitive neurons that receive and integrate thermal signals from the body

and induces thermoregulatory responses so as to maintain relatively constant core body temperature. In prenatal or early post-hatching ontogeny, during critical developmental phases lifelong epigenetic adaptation takes place (Tzschentke and Plagemann 2006). Thus by thermal manipulation (TM) the 'set point' can be altered resulting in change in the threshold for heat production as well as heat loss of an animal. In chicken, TM can be carried out during egg incubation (pre-hatching) and post hatch period. In the process of thermal manipulations during embryogenesis three factors should be considered namely the critical phase, the temperature level and duration of exposure (Yahav 2015). TM carried from 7th day of egg incubation till 18th day of incubation and during first 10 days post hatch has been shown to produce beneficial effects (reduced stress and lower mortality) when broiler type chicken are raised under hot environmental conditions. Higher temperature during incubation may result in poor hatchability (own unpublished data; Yahav et al. 2004). The hypothesis underlying TM is that cells exposed to non lethal higher temperatures activate cellular stress responses that confer thermotolerance and resistance to future heat stress related damages. Epigenetic modifications such as DNA methylation and histone modifications in developing chicken embryos were found to be relatively stable after hatching, indicating these alterations may play critical role in development of chick embryos and neonates (Li et al. 2015).

6.3.1 HSP in Thermal Manipulated Chicken

Pre-natal thermal manipulated coloured broilers reared and slaughtered at high ambient temperature had lower HSP gene and protein expressions in different tissues (Rajkumar et al. 2015; Rajkumar et al. 2017; Vinoth et al. 2015). There was breed differences, Naked Neck chicken had no or variable effect of TM in these reports. This may be due to lesser feather coverage in Naked Neck chicken that helped in dissipating excess body heat than the coloured broiler used in the experiment. The lower HSP genes expression in TM birds were analysed by luciferase reporter gene assay and found to be due to methylation at several sites in the promoter regions of these genes (Vinoth 2016). Furthermore, in the same study difference in overall methylation frequency of HSP70 due to TM between two breeds of chicken was observed. Heat conditioning of 5–7 day old chicks on subsequent thermal challenge at 42nd day had decreased HSP 27, 70 and 90 expressions in heart, liver and lung tissues (Yahav et al. 1997; Vinoth et al. 2016). It is clear from these reports that TM during incubation or at early age confers thermotolerance during adulthood and this thermotolerance is through lowered body temperature during stress period. Furthermore, lower HSP levels in thermotolerant birds indicate lesser damage to the cellular structures. Few reports state that adult chicken that were heat conditioned as chicks or thermal manipulated during incubation when heat stressed later had higher HSP gene expressions (Wang and Edens 1998; Al-Zhgoul et al. 2013). One possibility for the differences in HSP expression in stressed adult birds after TM during embryonic development may be due to different protocols or birds

used. Most of the TM reports are in broiler chicken since this protocol may be expected to be more beneficial to broilers because of their higher body mass and therefore comparatively greater predisposition to heat stress than layer chicken. A single report indicated beneficial effect of TM in layer breeders where TM manipulated birds had lower HSP 70 and HSP 27 gene expression in spermatozoa under hot ambient condition (Shanmugam et al. 2015). There is correlation between body temperature and induction of HSP, however, it was opined that the HSP response to early age TM was not part of long-term mechanism of this strategy (Yahav 2009). HSP response in acquisition of thermotolerance is only a part of the TM strategy and efforts in understanding other molecular mechanisms playing role are in progress. Peri-natal TM affects the development of certain tissues/organs in the adult stage. For example, in brain two genes (R-Ras3 and brain derived neurotropic factor) were found to be expressed higher in TM birds (Yahav 2009). Proteomic analysis revealed higher expression of UMP-CMP kinase enzyme in heat stressed TM birds (Vinoth 2016). This enzyme is involved in both denovo and salvage pathways of nucleosides and catalyses the synthesis of UTP, CTP and dCTP (Pasti et al. 2003).

6.4 Conclusions

Climate change and resultant increase in environmental temperature posses challenges in sustaining commercial chicken production and welfare. Heat stress causes reduction in production performance of both layer and broiler chicken. It is well established that in response to heat stress HSP expression is increased as a cellular mechanism to protect different protein against damage. During heat stress higher HSP expressions could be observed in almost all important body organs of chicken. Recent research is pointing to the advantages of epigenetic adaptation through thermal manipulation strategy. However, this strategy has to be fine-tuned for the temperature level and embryonic developmental stage so that the critical phases of different body control systems are beneficially modified. Furthermore, the interaction between HSP and other molecular systems needs detailed study for widespread commercial exploitation of thermal manipulation strategies in chicken.

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Part III
Aquatic Animals

Chapter 7
Heat Shock Proteins in Fish Health and Diseases: A Pharmacological Perspective

Kartik Baruah, Parisa Norouzitallab, and Peter Bossier

Abstract Disease outbreaks are considered one of the largest constraints for the sustainable development of the aquaculture sector. Though applications of antibiotics manage to control and prevent infectious microbial diseases, however, its extensive uses have also unavoidably resulted in the emergence of 'superbugs' that resist conventional antibiotics. This calls for the development of new approaches for combating infections. Recently, heat shock proteins have been suggested to mediate the generation of strong innate and adaptive immune responses against many diseases in plants and terrestrial animals, leading to the formulation of strategies to fight infections. In this review, the potential of a new treatment, heat shock protein-based therapy, for overcoming the menace of diseases in farmed aquatic animals of commercial importance are discussed.

Keywords Aquaculture · Cross protection · Disease · Heat Shock Protein · Immunity · Immunostimulant

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Abbreviations

APCs	Antigen presenting cells
DAMPs	Damage-associated molecular patterns
DC	Dendritic cells
HSC	Heat shock cognate protein
HSP	Heat shock protein
IFN	Interferons
IgA	Mmunoglobulin A
IgG	Mmunoglobulin G
IL	Interleukin
MHC	Major histocompatibility complex
TLRs	Toll like receptors
TNF	Tumor necrosis factor

7.1 Introduction

The farming of fishes and crustaceans remain important sources of food, nutrition, income and livelihoods for hundreds of millions of people around the world. World per capita fish supply reached a new record high of 20 kg in 2014, thanks to vigorous growth in aquaculture, which now provides half of all fish for human consumption. While the intensification of aquaculture has led to remarkable improvements in productivity, it has also led to disease epidemics, involving bacterial, fungal, viral and parasitic pathogens. Recently, organizations like FAO and European Union considered disease outbreaks as a significant constraint to the development of this sector worldwide as they cause great losses even up to 100%, with annual losses of billions of dollars. Therefore, there is urgency for controlling disease outbreaks for sustainability of the sector and to meet the growing demand for animal protein by the increasing human population.

Hitherto, the use of conventional approaches, such as antibiotics has achieved limited success in the prevention and cure of aquatic diseases as their repeated use has been questioned on account of increased bacterial resistance to drugs and persistence in the environment. Additional problems associated with the use of antibiotics include the limited number of antibiotics registered for use in aquaculture and possible residues in the resulting aquaculture products. The significant disadvantages of the use of antibiotics emphasize the need for alternative disease prevention techniques.

Disease prevention can be achieved by various ways. For instance, by introduction of specific pathogen free broodstock, optimization of feed, the improvement of the water quality, the avoidance of stress, and a good hygiene. In conjunction with proper health management, prophylactic treatments, such as vaccination and immunostimulation are indispensable tools for disease control in aquaculture (Defoirdt et al. 2004).

Vaccination has been effective in controlling many diseases affecting the aquaculture industry, but only in conjunction with good farm management, nutrition and other disease control methods. So far, various vaccines are being developed and marketed. However, they were unable to be used as a universal disease control measure in aquaculture. It is because there are situations when specific treatments may not be available or may be ineffective due to stress-induced immunosuppression or immaturity of the animal. Vaccination by injection is impractical when applied to small fish or large numbers of fish. This indicates that all the above-mentioned anti-infective strategies are not always effective in solving every problem alone. Hence, this called for the development of new approaches and technologies suited to improve the health of farmed species and, at the same time, not be harmful to the environment. In this review, the potential of heat shock protein-based therapy for overcoming the menace of diseases in aquaculture are discussed.

7.2 Heat Shock Proteins – What Are They?

The heat shock proteins (HSP), also called molecular chaperones, are evolutionary conserved proteins, of varying molecular weight (16–100 kDa) produced in all cellular organisms when they are exposed to cellular stress (Welch 1993). The initial observation that led to the discovery of the HSP was by Ritossa (1962), who observed that temperature shock induced an odd puffing pattern on polytene chromosomes of *Drosophila* salivary glands. Such swellings were recognized as indication that genes were being activated in those areas of the genome to give rise to their encoded proteins. These therefore became known as the 'heat shock loci'. It was not until 1974 that these loci were proven to be the sites of transcriptional induction of genes encoding for a particular group of proteins, which were designated as HSP. Although the response in the *Drosophila* was originally found to be up-regulated by exposure of their cells to heat, it is now recognized that the response is universal to all other eukaryotic and prokaryotic cells and that other stressors such as anoxia, ischaemia, toxins, protein degradation, crowding, starvation, hypoxia, acidosis and microbial damage also lead to their up-regulation (Roberts et al. 2010).

7.3 Induction and Regulation of HSP Expression

Intracellular counterparts of HSP, referred to as 'heat shock cognate (Hsc)', which are constitutively expressed, are also found in normal unstressed cells. They represent 5–10% of the total protein in healthy growing cells (Pockley 2003). It is now recognized that Hsc are universal to all cells and essential for various homoeostatic functions, including maintenance of protein structure and folding, supporting and repairing damaged cytoskeleton elements, assisting in the production and

folding of intracellular proteins, enzymes and hormone receptors and maintenance of mitochondria, and nuclear and cell wall lipoprotein membranes. When such (healthy) cells are stressed by various insults/stressors, there is up-regulation of the constitutive Hscs to produce newly formed HSP which can be detected in the cells at concentrations two or three times those of the constitutive chaperones. Thus, the term 'stress proteins' is also used to describe them (Locke 1997).

7.3.1 Categorization of HSP and Their Localization

HSP are categorised into several families that are named on the basis of their function, sequence homology and approximate molecular mass in kilodaltons (kDa). The families primarily include HSP110, HSP90, HSP70, HSP60, HSP47 and low molecular mass HSP. Among the various HSP, the HSP70 group of protein is the most conserved and the best studied class of HSP (Asea 2005). The different HSP are localised in various intracellular compartments (Table 7.1).

7.3.2 HSP and Cross-Protection

Cross-protection, also known as cross-tolerance, is the ability of one stressor to transiently increase the resistance of an organism to a subsequent heterologous stressor (Volker et al. 1992). Study of cross-protection in fish was initiated by the

Table 7.1 Families of HSP and their cellular localization

Family	Members	Monomer Mass (kDa)	Location
HSP110	HSP110 HSP105 Grp170	80–110	Cytosol, nucleus
HSP90	HSP90 α/β Grp94 / Gp96	82–96	Cytosol, nucleus, ER
HSP70	HSC70 HSP72 DnaK Grp75/mtHSP70 Grp78 / BiP	67–76	Cytosol, nucleus, mitochondria, ER
HSP60	GroEL HSP60 HSP65 Rubisco-binding protein	58–65	Cytosol, mitochondria, ER
sHSP	HSP10, HSP16 HSP20, HSP25 HSP26, HSP27 GroES, α-crystallin	18–40	Cytosol, nucleus,

demonstration that heat stress shields winter flounder cells against deleterious effect of chemical exposure (Brown et al. 1992). Although the underlying causes are unclear, however, HSP are thought to be involved in the protection as cross-tolerance was associated with the induction of HSP28, HSP70 and HSP90 proteins. In another example, thermal shock at 26 °C for 15 min confers protection against osmotic shock at 45 g/L in salmon (Dubeau et al. 1998), a situation where smolts exhibiting elevated branchial and hepatic HSP70 consistently display highest survival. These studies indicate that aquatic organisms acquire enhanced protection against environmental change by regulating endogenous HSP. The mechanism by which HSP initiate cross-protection to abiotic stressors is uncertain, but may be by 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 Linquist 1993). Collectively, these results suggest that this cross-protection might be a useful tool to protect fish to various upcoming stressful events.

7.4 HSP and the Immune Response

Initially, HSP response to cellular stressors was considered a short-term functional response, with a range of essentially housekeeping and cytoprotective functions (Pockley 2003). However, now it is becoming clear that the HSP response also plays a significant role in regulation of the immune response of both vertebrates and invertebrates (Baruah et al. 2010; Ryckaert et al. 2010).

7.4.1 Endogenous HSP as DAMPS

In 1992 Janeway (1992) proposed a theory concerning the crucial event controlling the initiation of an immune response. In this ‘stranger’ model, it was stated that to mount an immune response, discrimination has to be made between infectious non-self (e.g. bacteria) and non-infectious self by antigen presenting cells (APCs). The author postulated that APCs have pattern recognition receptors (PRRs) that can recognize conserved features of a pathogen, called pathogen-associated molecular patterns (PAMPs). When PAMPs were present, APCs get activated and start to produce co-stimulatory signals, process foreign antigens and present them to passing T-cells. This theory, however, could not explain several immunological phenomena, such as autoimmunity and transplant rejection. Considering these exceptions, Matsinger proposed the ‘danger’ theory in 1994, which is based on the idea that an immune response is ultimately controlled by recognition of the damage induced by a pathogen, rather than the pathogen itself. According to this, abnormal cell death is a potential threat to the organism, whether it is caused by an infection or other pathological processes, and it could be a universal sign of danger. Danger

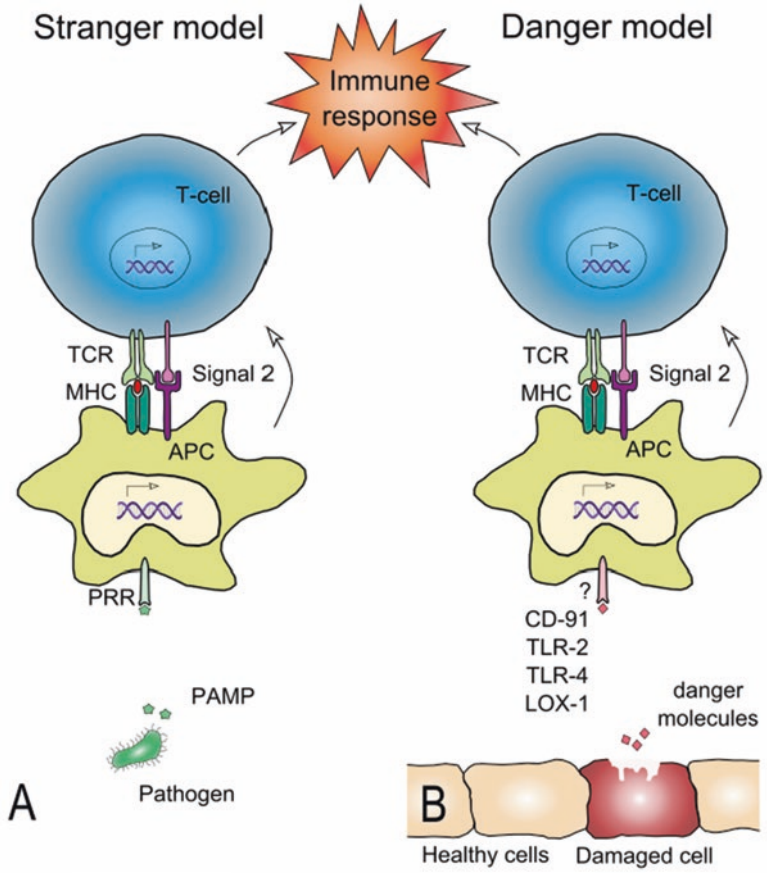

Fig. 7.1 The stranger (**a**) and danger (**b**) model of immune stimulation. The stranger model states that APCs express a set of receptors (PRRs) that allow APCs to discriminate between 'infectious non-self' and 'non-infectious self'. Recognition of conserved PAMPs activates the APCs to up-regulate costimulatory signals, to process antigens and present them to T-cells. The danger model suggests that immune activation is initiated by the damage a pathogen induces rather than the by pathogen itself. Necrotic, infected or damaged cells release intracellular molecules (called danger signals) that activate APCs to provide costimulatory signals for the initiation of an adaptive immune response. Thus, danger signals indicate a dangerous situation and allow the initiation of immune response. Putative receptors for HSP are CD-91, TLR-2, TLR-4 and LOX-1 (Binder et al. 2004). TCR = T-cell receptor (Osterloh & Breloer 2008)

signals (or damage-associated molecular patterns, DAMPs) are considered to be conserved, abundant and ubiquitously expressed self molecules that are normally hidden within the cell in healthy tissue, but that can be released from distressed, infected, injured or necrotic cells (Fig. 7.1). It has been suggested that in reality both models are correct, hence that both PAMPs and DAMPs can alert the immune system independently and that there is possibly even an overlap between the two models, such that stress-induced endogenous proteins form complexes with

microbial products, and these complexes are recognized by APCs (Kono and Rock 2008).

As stated above, HSP are normally intracellular. These endogenous HSP are potential immunological danger signals (Moseley 1998), as they are up-regulated under various conditions of stress, and are release by stressed, infected, necrotic or tumour cells, but not from apoptic cells (Basu et al. 2000). In mammals, it has been shown that after release, these extracellular HSP can induce the production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 and chemokines (Gao and Tsan 2004) in APCs, such as dendritic cells (DCs) and macrophages. A study, performed by Campisi et al. (2003) reported that intense stressor exposure increased extracellular HSP72 in mice, which acts as a danger signal to potentiate the NO response to bacterial challenge and facilitate recovery from bacterial inflammation. Several other studies have demonstrated that extracellular endogenous HSP can trigger the translocation of nuclear factor (NF)-κB to the nuclei of macrophages and dendritic cells (Valentinis et al. 2008).

In farmed aquatic animals, the effects of extracellular endogenous HSP, especially from immunological perspective, have not been studied in a comprehensive manner. However, because HSP are among the most ancient and highly conserved proteins, some researchers hypothesized that extracellular endogenous HSP will also serve as danger signals and activate the innate and/or adaptive immune responses within animals, and defending the animals against (pathogenic) stress. This assumption was supported with the findings of a study in invertebrate (which unlike vertebrates have only an innate immune response), brine shrimp *Artemia franciscana*, where a non-lethal heat shock (NLHS) of 37 °C for 30 min followed by 6-h recovery period was found to increase HSP70 expression in the shrimp. This NLHS shrimp when challenged with pathogen (e.g. *Vibrio harveyi, V. campbellii* or *V. proteolyticus*) had higher percentage of survival than the un-shocked controls (Sung et al. 2007; Baruah et al. 2010). Similarly, a combined hypo- and hyperthermic stress followed by recovery at ambient temperature safeguarded *Artemia* larvae against *V. campbellii* infection. Interestingly, enhanced survival was associated with the induction of HSP70 (Sung et al. 2008). However, hypothermic stress or osmotic stresses which did not induce HSP70 failed to increase *Artemia* tolerance to *Vibrio* challenge, indicating the possible role of this protein in conferring resistance against vibriosis (Sung et al. 2008). However, it can be argued that during exposure to stressors, along with HSP70, other member of HSP might also be induce, the observed protective effects could be due to the combined or synergistic effects of the constellations of HSP. In a further study, the work of the above authors was extended by Baruah et al. (2010), with the aim of determining the biological functions (in terms of *Vibrio* protection) of HSP70, and the results showed that recombinant *Artemia* HSP70, when overproduced in *E. coli* and fed back to *Artemia* markedly protected the shrimp against a subsequent vibrio challenge, and interestingly phenoloxidase (one of the major component of the crustacean immune system) expression and activity was induced when HSP70 was fed to *Artemia* (Baruah et al. 2010). This result indicates that HSP70 possesses pharmacological ability and has significant potential for application in farmed aquatic animals.

7.4.2 *Exogenous HSP as Antigens and Elicitors of the Immune System*

Accumulating evidence suggested that HSP represent major antigens of many pathogens (Zügel and Kaufmann 1999). The stress imposed by the host on its pathogenic aggressors may lead to increased HSP synthesis. Due to their abundance, HSP become prominent antigens that instigate a major portion of the immune repertoire. Members of the HSP60 and HSP70 families represent major targets for antibodies in many infections with helminths, protozoa and bacteria (Young et al. 1990). Recent years have seen the emergence of compelling evidence that HSP possess unique properties that permit their use in generating specific immune responses against infectious diseases (Srivastava et al. 2009). In vitro experiments indicated that exogenous HSP (e.g. bacterial HSP70 DnaK and HSP60 GroEL), can induce immune cells to express and secrete cytokines, such as TNF-α, IL-1 and IL-6 (Tabona et al. 1998), and adhesion molecules, such as E-selectin and intercellular cell adhesion molecule (ICAM)-1 (Galdiero et al. 1997), and that they can activate NF-κβ (Kol et al. 1999) and the Toll/IL-1 signaling pathway and thus mount an immune response against infection (Vabulas et al. 2001). A study conducted by Oladiran and Belosevic (2009) had shown that recombinant HSP70 from *Trypanosoma carassii*, an extracellular protozoan that lives in the vascular system of cyprinid and non-cyprinid fish, could activate goldfish macrophages and stimulate the production of pro-inflammatory cytokines, such as interferon (IFN)-γ, TNF-α, IL-1β and IL-12. The parasite HSP70 could also up-regulate the expression of inducible NO synthase and induced a strong NO response of goldfish macrophages.

HSP from Mycobacterium tuberculosis were the first exogenous HSP that generated interest as potential protective antigens, largely because of their importance in the immune response in mice and humans (Young et al. 1990; Mehra et al. 1992). Among the different HSP, the HSP60 family members have been reported to be highly immunogenic in the case of mycobacterial infections. In fact, before identification as a member of the HSP60 family, HSP60 was known as the common antigen of Gram-negative bacteria (Thole et al. 1988). As early as 1986, it was already demonstrated that HSP60 from *M. bovis* constitutes an immunodominant antigen for infection, which could stimulate cellular and humoral immune responses by their specific recognition by T-lymphocytes (Emmrich et al. 1986). It has been also reported that HSP70 derived from Toxoplasma gondii induced DC maturation and stimulated IL-12 responses (Kang et al. 2004). Another example of a protective anti-HSP immune response has been shown in murine infection with *Yersinia enterocolitica*. Immunization of mice with *Yersinia*-HSP60 induced a strong *Yersinia*-HSP60-reactive response which conferred protection against a challenge with *Yersinia* (Noll and Autenrieth 1996). In another study, mice, which received plasmid DNA encoding for mycobacterial HSP60 were partially protected against subsequent challenge with *M. tuberculosis* (Bonato et al. 1998; Lowrie et al. 1995).

In aquaculture animals, there are good evidences that induction of HSP(s) enhances tolerance to other subsequent abiotic and pathogenic biotic stressors and

various strategies to inducing HSP have been studied (Roberts et al. 2010). Normally, for induction of HSP, exposure of an aquatic animal to some form of stressor is a prerequisite. The stressor used has generally been thermal shock per se because of the ease of application. However, recently, several novel methods for inducing HSP within the host have been developed, and these include supplying exogenous prokaryotic or eukaryotic HSP derived from bacteria or animal (e.g. brine shrimp *Artemia*), respectively or by supplying exogenous natural HSP stimulation factor.

Having demonstrated that induction of endogenous HSP70 in the *Artemia* host was concomitant with enhanced resistance to *Vibrio* challenge, Sung and co-workers adopted the novel strategy of feeding the brine shrimp with *Escherichia coli* which had been previously heat-shocked to induce them to overproduce different prokaryotic HSP. These induced *E. coli* cells were shown to enhance *Vibrio* resistance in the *Artemia* and feeding of the E. coli was found to correlate with high levels of DnaK, the 70 kDa bacterial HSP within the E. coli. They suggested therefore that the feeding of HSP overproduced in non-pathogenic *E. coli* might be a viable alternative to use of antibiotics to control vibriosis and other bacterial infections in *Artemia* (Sung et al. 2009a, b). In another study, Baruah et al. (2010) overproduced HSP70 derived from *Artemia* in *E. coli* and then fed this exogenous *Artemia* HSP70 producing *E. coli* cells to *Artemia* followed by challenge with *V. campbellii*. After 36 h of challenge, these authors found that *Artemia* fed with recombinant *Artemia* HSP70 protein had higher survival than that of those fed with control *E. coli* cells not-producing *Artemia* HSP70 protein. Interestingly, these authors found an increase in the phenoloxidase activity (an important component of the innate immune system of invertebrates) of *Vibrio*-challenged *Artemia* fed with *Artemia* HSP70 proteins, suggesting that the *Artemia* HSP70 protein conferred protection to *Vibrio*-challenged *Artemia* by inducing the prophenoloxidae innate immune system (Baruah et al. 2010).

The use of exogenously heat shock-stimulated (non-pathogenic) bacteria as a novel therapeutant was further examined in the platyfish, *Xiphophorus maculatus*, by Ryckaert et al. (2010). In this study, the use of host-derived and non-host derived HSP against bacterial diseases was investigated using the platyfish-*Yersinia ruckeri* host-pathogen model. In this infection model, the effect of different treatments with HSP on the survival of the fish after bacterial infection was tested: non-lethal heat shock (NLHS), intracoelomal injection with two recombinant bacterial HSP, GroEL and DnaK, and a combination of a NLHS and an injection with bacterial HSP. The results showed that a NLHS could not confer protection to the fish against a subsequent infection with *Y. ruckeri*. However, when the fish received an injection with bacterial HSP, *Y. ruckeri*-induced mortality was reduced. This effect became significant when the administration of bacterial HSP was combined with a NLHS.

Recently, a commercial formulation TEX-OE® - a patented extract of the skin of the prickly pear cactus, *Opuntia ficus indica*, which can act as a non-stressful precursor for induction of high levels of endogenous or host-derived HSP in animal tissues, has become available for use in fish and shellfish. It readily causes prestress conditioning of the animal without any apparent hazardous effect. When it was applied to prestimulate fish before exposure to abiotic or pathogenic biotic stressors,

increased HSP levels were detectable after the first sign of stress, with little or no delay. Studies on the effect of prestimulation of salmon and gilthead sea bream, *Sparus aurata* with TEX-OE® before exposure to *V. anguillarum* infection have shown that when laboratory infections are induced at the LD50 level, mortality in groups of fish which have been exposed to TEX-OE® prior to infection was generally less than half those occurring in infected controls (El Fituri 2009). Based on the findings of the above-mentioned studies, it is apparent that HSP proteins are strongly immunogenic and may function well as a pharmacological agent in the treatment of diseases in farmed aquatic animals of commercial importance.

7.5 HSP as Vaccine for Use in Aquaculture

First steps towards the possible use of bacterial-derived HSP in vaccine development for application in aquaculture animals have already been set. Wilhelm et al. (2005) successfully used a formulation that is comprised of recombinant HSP60 and HSP70 of *Piscirickettsia salmonis* as a vaccine in Atlantic salmon against a subsequent infection with this pathogen. Intracoelomal injection with 20 μg of recombinant HSP elicited a humoral response and resulted in a 92% survival in the vaccinated group compared to 23% in the control group. In a second study using Atlantic salmon, a formulation which consisted of *P. salmonis* HSP60, HSP70 and flagellar protein FlgG was shown to be efficacious as a recombinant vaccine (Wilhelm et al. 2006). Additionally, another member of the HSP family, named ChaPs, was also shown to be a good candidate as a vaccine component to control outbreaks of salmon rickettsia syndrome (Marshall et al. 2007). *Flavobacterium psychrophilum* HSP60 and 70 were recently identified as possible antigens for vaccine development (Sudheeshl et al. 2007). In a subsequent study, these proteins were used as a recombinant and DNA vaccine against coldwater disease in rainbow trout (Plant et al. 2009). Only fish immunized with recombinant HSP70 showed marked increase in the antibody levels against *F. psychrophilum*. However, protection against *F. psychrophilum* challenge was not observed in any of the treatments. The authors listed the following possible reasons for the non-protective effect of the formulation: 1) the fish received a total of 8 μg of recombinant protein, which is substantially less than the total of 30 μg used in the study by Wilhelm et al. (2006). 2) the recombinant HSP contain an additional V5 tag, which enables the detection of the fusion protein with an anti-V5 monoclonal antibody. The authors suggest that this tag may interfere with the function of the recombinant HSP. It can be concluded from these studies that pathogen-derived HSP are ideal as vaccine candidates for use in aquaculture.

7.5.1 HSP as Adjuvant in Vaccines

Many of the recombinant or synthetic antigens now being considered as vaccine candidate antigens are not sufficiently immunogenic by themselves to induce a strong and protective immune response. To improve the immunogenicity of a vaccine, the addition of an adjuvant is required. An adjuvant is any substance that augments immunity to an antigen with which it is mixed. HSP are valuable candidates to be used as adjuvant because they enhance immune responses when covalently linked to synthetic peptides (Suzue and Young 1996). The immunogenic properties of HSP came to light primarily because of their ability to induce anti-tumor immunity. This property, first described for the endoplasmic reticulum-resident glucose related HSP Gp96 was subsequently also observed for HSP70 (Udono and Srivastava 1993), HSP90 (Udono and Srivastava 1994), HSP170 and HSP110 (Wang et al. 2001). Additionally, a study from Heikema et al. (1997) demonstrated that Gp96 preparations isolated from influenza virus-infected cells have been shown to be protective against a challenge with the influenza virus. Taken together, these studies demonstrated that HSP-peptide complexes purified from tumors or cells infected with pathogenic agents are associated with the antigenic epitopes of those agents and that such HSP-peptide complexes are immunoprotective against the cognate infectious agents.

The unequivocal demonstration that the specific immunogenicity of tumor- or infected cell derived HSP-preparations is elicited by HSP-associated peptides raised the question whether immunogenic HSP-peptide complexes could be generated in vitro. To verify that, Blachere et al. (1997) reconstituted Gp96- and HSP70-peptide complexes in vitro using a panel of seven peptides, and they showed that HSP alone and peptides alone were non-immunogenic. However, HSP-peptide complexes elicited MHC I restricted antigen-specific CD8+ T cells. These results suggested that HSP-peptide complexes elicit CD8+ responses in spite of exogenous administration, a phenomenon which is called cross-presentation. Exogenous antigens are typically routed through the MHC II presentation pathway and elicit CD4+ responses, whereas endogenously synthesized antigens are presented through MHC I molecules and stimulate CD8+ cells (Townsend and Bodmer 1989). The ability of HSP-peptide complexes to cross-present is significant from the vantage point of their potential use as vaccines against intracellular infections, where CD8+ responses have a protective value (Srivastava and Amato 2001). In this regard, Matsutake and Srivastava (2000) have made the observation that immunization with HSP-peptide complexes also elicits an antigen-specific MHC II restricted CD4+ response.

HSP-APC interaction has two distinct consequences (Fig. 7.2). Firstly, the HSP-peptide complexes are taken up by the APCs and the peptides are represented on MHC molecules of the APCs. Secondly, HSP can, regardless of the chaperoned peptides, induce the secretion of inflammatory cytokines, chemokines, and NO by macrophages and DCs, and they can up-regulate the expression of costimulatory molecules on DCs (Basu et al. 2000). These effects involve receptor engagement, signaling, and translocation of NF-αB to the nucleus of macrophages and DCs.

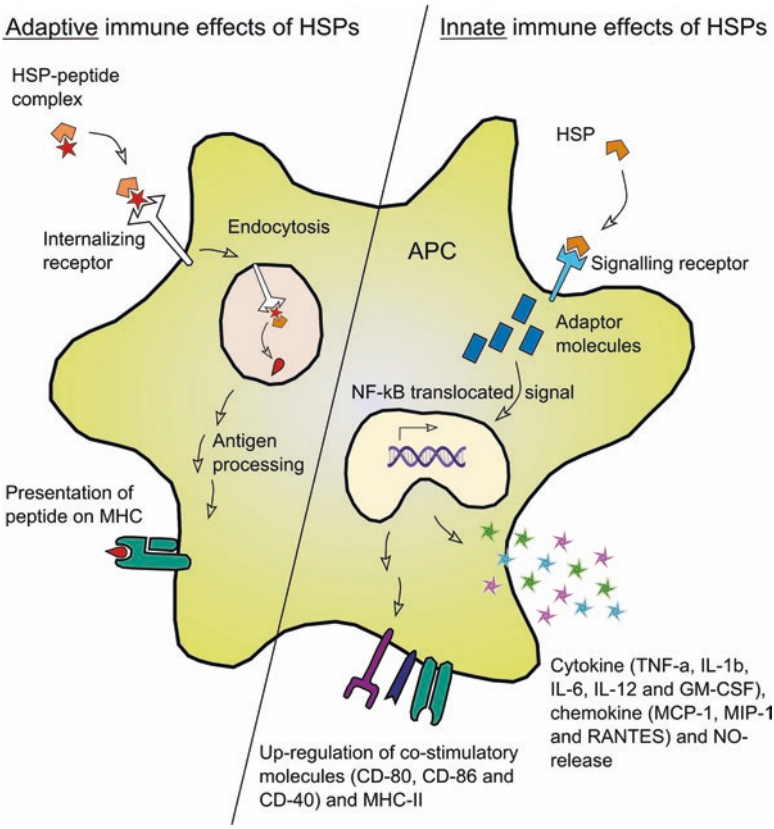

Fig. 7.2 Schematic presentation of the interaction of HSP with APCs through specific receptors (Todryk et al. 2003)

The HSP-chaperoned peptides enter APCs through specific receptors such as Toll-like receptors (TLRs), scavenger receptors (lectin-like oxidized LDL receptor) and/or CD91 (Binder et al. 2004), and induce T-cells by increasing antigen display by MHC class I and II molecules (Srivastava 2002). Independent of the chaperoned antigens, HSP-APC interaction leads to up-regulation of co-stimulatory molecules on APCs and secretion of proinflammatory cytokines or chemokines (Singh-Jasuja et al. 2000). TLRs 2 and 4 and the downstream myeloid differentiation primary response gene (MyD88)/NF-αB pathway have been proposed to mediate the HSP triggered DC activation (Asea et al. 2002). Other receptors, including CD40 are also potentially involved in transducing activation signals (Wang et al. 2002).

In addition to studies with endogenous HSP, a number of interesting observations have been made with exogenous HSP-peptide complexes. In fact, exogenous HSP70 can be fused or covalently linked with peptides to elicit specific immunity to tumours or viruses (Ciupitu et al. 1998). It has been shown that covalent complexes of

mycobacterial HSP65 or HSP70 and peptides could be used to elicit potent and specific anti-peptide antibodies, without the use of additional adjuvants (Barrios et al. 1992). Lehner et al. (2000) showed that M. tuberculosis-derived HSP70 alone could stimulate the production of β-chemokines in T-cells in nonhuman primates. When the HSP were linked to antigenic peptides, these complexes combined the generation of β-chemokines with an adjuvant function by enhancing specific T cell proliferative responses and immunoglobulin (Ig) G and IgA antibodies. The authors suggested that exogenous HSP can stimulate β-chemokine production which may be responsible for innate adjuvanticity. The up-regulation of β-chemokines enables DCs, macrophages, CD4+ and CD8+ T cells to be attracted (Luster 1998). In fact, the adjuvant effect of HSP70 may be accounted for by the induction of β-chemokines which attract the essential cellular components to the antigens, thereby enhancing the immune response.

Hitherto, no studies have been conducted on the use of HSP as vaccine adjuvants in aquaculture. Nonetheless, the data presented here demonstrate that these proteins are potential candidates. HSP can be of special importance in the development of vaccines against viruses or intracellular bacteria, as they are able to shuttle the antigens into the endogenous antigen processing pathway.

7.6 Conclusions

In farmed aquatic animals, research on HSP and their pharmacological properties is gradually progressing. However, based on the promising findings from medical and veterinary research, it could be claimed that HSP show potential as immunostimulant and as a vaccine component. Additionally, accumulating evidence demonstrates that these proteins play key roles in aquatic organism growth and survival, ranging from stress tolerance to immune enhancement. In essence, overall results presented here suggest the potential of HSP in the development of a new generation of prophylactic treatments in aquaculture animals.

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Chapter 8
Physiological Role of Heat Shock Proteins, Molecular Function and Stress Removal in Fishes

Shib Sankar Sen and Sib Sankr Giri

Abstract Depending on the size, the specific locations and physiological roles of molecular chaperones differ within the cell. However, to tolerate stress HSP drives molecular mechanisms in animals. These proteins interact with multiple systems in diverse ways regulated by the endocrine system. HSP have crucial physiological roles in fish. The important role of HSP is in thermo tolerance as well as tolerance to cytotoxic effects of environmental contaminants and other stressors which are non thermal. HSP enhances immune response through both intra- and extra-cellular activities. Similarly, many studies have proved that most HSP are molecular chaperones which funtion for other cell proteins, and have better cytoprotective effects. To know more about factors, both biotic and abiotic regulating heat shock proteins data has been collected rapidly. However, earlier reports are focused on the role of HSP in development and their importance in fish in nature. Functional genomic approaches will provide the tools necessary to gain a comprehensive understanding of the significance of heat shock proteins in the cellular stress response, in the physiological processes. Heat shock proteins are considerably adaptable and potent molecules, the significance of which to biological procedures is highlighted by the high degree to which their structure and function are phylogenetically preserved. Our knowledge regarding physiological role of heat shock proteins is presently partial; though, a better understanding of their function and thus the skills of the capacity to control their power may lead to their use as therapeutic agents.

Keywords Heavy metals · HSF · HSP · Immunoregulation · Pathogen · Thermal window · UV radiation

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Abbreviations

ATP	Adenosine triphosphate
GAV	Gill-associated virus
Hsc	Heat shock cognates
HSF	Heat shock transcription factors
HSP	Heat shock proteins
HSR	Heat shock response
LPS	Lipopolysaccharide
MT	Metallothionein
TSD	Temperature-dependent sex determination
UV	Ultra violet
WSSV	White spot syndrome virus

8.1 Introduction

Heat shock proteins are a family of highly conserved cellular proteins present in all organisms including fish. HSP are found in virtually all living organisms, from bacteria to humans. HSP are involved in the folding and unfolding of other proteins. HSP that functions as molecular chaperone are classified according to their molecular weights. They are vitally involved in maintaining protein homeostasis and cell viability. HSP70 family of chaperones represent one of the most ubiquitous classes of chaperones found not only in eukaryotic cytosol, membranes, chloroplasts, ER and mitochondria but also in the extracellular molecules and in bacteria and certain archaea where they fulfil specific organellar- or tissue-specific functions (Kampinga et al. 2009; Ehrnsperger et al. 1997; Kappe et al. 2001). HSP70 depend on their ATP-regulated ability to interact with exposed hydrophobic surfaces of proteins for cellular activities.

When cells are exposed to elevated temperatures or other stressors, their expression usually increase (De Maio 1999). This increment of expression regulates transcriptionally. The dramatic upregulation of the heat shock proteins is a key part of the heat shock response and is induced primarily by heat shock factor (HSF) (Wu 1995). Heat shock proteins (HSP) play a pivotal role in protein homeostasis and cellular stress response in vivo (Feder and Hofmann 1999; Iwama et al. 2004; Multhoff 2007; Keller et al. 2008). According to the the degree of homology and the molecular size HSP are divided into several groups (families) of different molecular weights (kDa): e.g., HSP100, HSP90, HSP70, HSP60, HSP40, and small proteins (Lindquist and Craig 1988; Hallare et al. 2004; Park et al. 2007). These families of HSP play vital roles in physiological processes such as protein chaperoning activity, protection against apoptosis, steroid genesis, and stress tolerance. The heat shock response for maintaining cellular homeostasis following sublethal noxius stimuli is an evolutionarily conserved mechanism (Lindquist and Craig 1988). Several HSP

mediate the correct assembly and localization of intracellular and secreted polypeptides and oligomeric protein structures. The importance of HSP in the protein folding pathway is revealed in the fact that a number of heat shock genes are expressed at high levels during normal cell growth. Oxygen radicals, toxicants, and inflammatory stress increased the synthesis of HSP and often give rise to an accumulation of denatured and unusual folded proteins within the cell. Thus, the interaction of HSP with misfolded proteins during stress is thought to be an extension extra role under normal, non-stress conditions (Hightower et al. 1994).

Fish are ectotherms, meaning that their body temperatures adapt to surrounding water temperatures. Experiments proved that fish physiology and behavior are highly affected by water temperature (Olla et al. 1978; Portner and Farrell 2008). Increases in temperature beyond thermal window restricts the capacity of circulatory and ventilatory systems to fulfills oxygen demands, which resulting a decrease in the animal's aerobic activity (Brett 1971; Nilsson et al. 2009; Eliason et al. 2011). This reduction in aerobic activity is crucial for biological functions such as growth, reproduction, muscular activity, and behavior (Portner and Knust 2007; Portner and Farrell 2008). Further temperature increases lead to growth arrest, anaerobic respiration, protein misfolding, permanent inactivation of enzymes, and eventually apoptosis (Katersky and Carter 2007; Portner and Knust 2007; Wang and Overgaard 2007). Ectotherms in the wild usually utilize thermoregulatory behavior to avoid extreme temperatures and minimize acute changes in body temperature. Fish may also respond to temperature changes due to acclimatization, which involves recompensation in physiological parameters. A little has been studied in post-transcriptional regulation of HSP production (Silver and Noble 2012). It has been reported that post-transcriptional regulation of the heat shock response (HSR) plays a role in HSP accumulation in vertebrates (Silver and Noble 2012), e.g., due to high pressure in chondrocytes (Kaarniranta et al. 1998). Furthermore, post- transcriptional regulation of the HSR between differenc cell types in mammals have been reported (Kaarniranta et al. 2002).

Organisms can adopt variations of environmental temperatures by modification of their molecular and cellular structures (Somero 1995; Peck 2011). One of the major molecular event that is stimulated in a cell under thermal stress is the production of HSP. The HSP70 family has been most studied in its constitutive (HSC70) and inducible (HSP70) forms (Morimoto 1993). As molecular chaperones, HSP stabilize denaturing proteins and refold those that have already been misfolded and denatured (Hartl and Hartl-Meyer 2002; Tomanek 2010), thus, the HSR could be considered an ecologically and evolutionarily important factor in thermal adaptation, thermal tolerance limits (Feder and Hofmann 1999; Sorensen and Kristensen 2003; Tomanek 2010). Nevertheless, physiological responses due to HSR are not uniform among different taxa. For example, it has been demonstrated that some species of freshwater organisms are capable of generating a thermal stress response, while others could not generate HSR or simply do not have the necessary physiological mechanisms to generate this response (Clark and Peck 2009).

HSP are specifically generated when cells are exposed for a while beyond than their normal growth temperature. HSP was named as stress protein because they can

also be induced by oxidants, toxins, heavy metals, free radicals, viruses, and other stressors (Ponomarenko et al. 2013), and the term "heat shock protein" was coined by Tissieres et al. (1974). HSP showed different variations in their expression in most of organisms under a thermal window (Mahmood et al. 2014). Another common cytosolic chaperone HSP90 which is incapable to perform the refolding of denatured proteins participates in arranging the folding of various proteins, Accordingly, other chaperones, for instance HSP70, are necessary to complete this target (Vogel et al. 2006). On the otherhand HSP60 plays an important role in the protein-folding system and can disturb cellular homeostasis (Cechetto et al. 2000; Seveso et al. 2014). The role of HSP60 as an important component in the development of inflammation and immunity in response to bacterial and viral infections in shrimp has been studied (Huang et al. 2011). The ability of organisms to secrete HSP in response to environmental challenges has been broadly recognized (Parsell and Lindquist 1993; Feder and Hofmann 1999).

8.2 Discovery

Ferruccio Ritossa originally described heat shock proteins (HSP) on the fruit fly *Drosophila melanogaster* in the early 1960. After exposure to different kinds of stress such as heat shock in any cellular organism, expression of HSP was found and could be demonstrated subsequently (Reports and Kumar 1962). Other stress conditions, including heavy metals, hypoxia, nutrient deprivation and irradiation as well as oxidative and toxic stress, infections and exposure to inflammatory cytokines are also able to stimulate HSP expression (Reports and Kumar 1962; De Maio et al. 2012). Members of the HSP70 family were upregulated in bacteria in response to cellular stress, identified for the first time (Tissieres et al. 1974).

8.3 Size Dependent Classification and Functions of HSP

Molecular chaperones are ubiquitous and found within cellular environments. The main function is to develop defense mechanism against outer environment. The range of HSP families are made up by range from 10 to greater than 100 kDa in molecular size, and are situated in various cellular compartments show in Table 8.1. Depending on their molecular size the stress proteins typically are named. Heat shock and other forms of pathophysiological stress induce the expression of hsp in all cells and tissues Table 8.2. (Jonak et al. 2006; Simone et al. 2010). Many of the stress proteins are expressed constitutively and present continuously, and other proteins are expressed by stress (stress inducible). Main families of Hsp comprise small chaperones and ubiquitin, HSP10–30, HSP40–60, HSP70, HSP90 and HSP100.

HSP functions with co-factors together in complexes, to facilitate the proper folding and activation of many cellular proteins. Each of thesHSP is activated by

Table 8.1 The location size and role of HSP families

Molecular size (kDa)	Location	Function/Remarks	Reference
HSP 10–30	Mitochondria	Mitochondria plays a role in protein folding supplied by adenosine triphosphate (ATP)	Whitley et al. (1999), Meyer et al. (2003)
HSP 15–30	Plasma membrane	Embryonic cardiac muscle development, liposomal membrane protection	Ke et al. (2011), Rosenfeld et al. (2013), Juo et al. (2016)
HSP 20, 22, 27	Ubiquitous high in heart striated and smooth muscles	Chaperone activity stabilization of cytoskeleton, anti-apoptotic and anti-oxidant function	Bakthisaran et al. (2015)
HSP 40–60	Endoplasmic reticulum	Caring protocollagen, chaperoning intermediate filament	Jee (2016)
HSP 58–65	Mitochondria, chloroplasts	Chaperonin, assembly of oligomeric Proteins and folding of monomeric Proteins; high concentration required For growth at elevated temperature	Lindquist (1992)
HSP 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	Ogawa et al. (2011)
HSP 70	Mitochondria, cytoplasm, nucleus, endoplasmic reticulum	Associated with reducing infarct area by ischemic brain injury and protection against apoptotic death	Jackson (2013)
HSP 82–96	Cytoplasm	Essential for viability; increased Concentration required for growth At high temperatures	Hawkins et al. (2008)
HSP 100–110	Cytosol and nucleus	Helping immune response	Dimauro et al. (2016)

Note: *HSP* heat shock protein.

phosphorylation which is catalyzed by a specific kinase to involve in signal pathway (Fig. 8.1). Cellular homeostasis is maintained by the newly synthesized stress proteins under such conditions by enhancing correct folding of stress-accumulated misfolded proteins, preventing formation of protein aggregates and inducing proteolytic degradation of misfolded or denatured proteins. In both upstream and downstream of the mitochondrial events, HSP can also play pro-apoptotic or anti-apoptotic

Table 8.2 Triggers of the cellular stress reaction

HSP stress	Cells and tissues
Environmental stressors	Hyperthermia
	Heavy metals
	Amino acid
	Inhibitors of energy metabolism
Oxidative stress	DNA damage
	Weaking carbohydrate, lipid, protein metabolism
	Apoptosis
ER stress	Glucose deprivation
	Inhibition of protein glycosylation
	Disturbance of calcium homeostatis
	Hypoxia
Pathological processes	Viral and pathogen infection
	Fever
	Inflammation
	Ischaemia
	Oxidative injury
Physiological processes	Cell cycle
	Growth factors and cytokines
	Embryologic development and differentiation

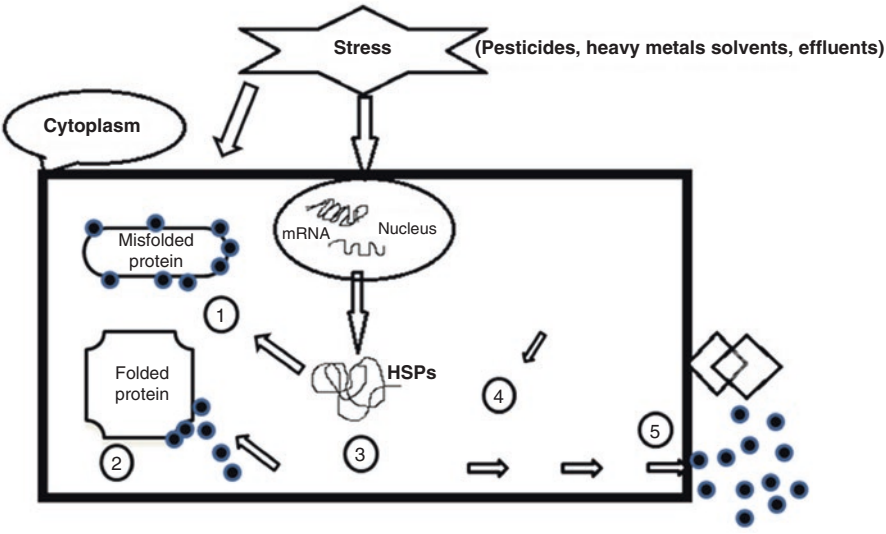

Fig. 8.1 Schematic represenatation of HSP induction under environmental stress conditions: 1. Correct folding of stress accumulated misfold proteins, 2. Prevention of protein aggregation, 3. Proteolytic degrardation of misfolded or denatured proteins, 4. Anti-apoptotic/pro-apoptotic properties, and 5. Re-establishment of cellular homeostasis

function by interacting with various proteins of apoptosis pathway. HSP10 (chaperonin) suppressed maternal immune response via releasing from fetal placental unit (Noonan et al. 1979) and thus considered as suppressor. HSP10 contains 101 amino acids and is used as a biomarker in endometrial cancer (Dube et al. 2007). HSP 10 in mitochondria plays a role in protein folding in presence of adenosine triphosphate (ATP) Table 8.1. (Meyer et al. 2003). HSP10 is produced as a by-product during the process of neoplastic cell proliferation which acts as a growth factor in the cell (Quinn and Morton 1992). sHSP are related with molecular size 15–30 kDa HSP and it has been know that there are 9 sHSP in mammals (Mounier and Arrigo 2002): Hsp27 (denoted Hsp25 in mice), αA- and αB-crystallins, HSP20, HSPB2, HSPB3, cvHSP or HSPB7, HSP22 or HSPB8, and HSPB9. Human sHSP has 105–205 amino acids and α-crystallin domain as a homologous 80 sequenced residues (Ingolia and Craig 1982). This domain shows highly conserved structure and 38–60% of amino acid identity (Mounier and Arrigo 2002).

Cytosolic HSP40 has forty-three kDa sized HSP has various amino acidic sequences that results in different N-terminal length. Those HSP also comprise conserved α-crystallin core domains and C-terminal extension domain including highly conserved I-X-I/V motif (Ghosh et al. 2005). All cells expressing type I procollagen also express HSP47. Stimulation of the expression of HSP47 mRNA in smooth muscle cells occurred by heat shock and oxidized low density lipoprotein. These findings identify HSP47 as a novel constituent of human coronary atheroma, and selective upregulation by stress raises the possibility that HSP47 may be a determinant of plaque stability. HSP47 an endoplasmic reticulum molecular chaperon and functions to support collagen folding and its release to extracellular environments. HSP56 is located in the cytosol. HSP60 forms a large (970 kDa) hetero-oligomeric protein complex called the TCP1 ring complex which is essential for protein assembly. HSP60 is located in mitochondria.

The family of GRP78, HSP70 is in the cytosol which plays role in helping protein folding assembly and refolding, transporting and blocking protein degradation in endoplasmic reticulum. HSP90 is divided into HSP90 α and HSP90 β. HSP90s are located in cytoplasm and endoplasmic reticulum. HSP90 α plays key role in myosin folding and sarcomere formation (Du et al. 2008; Hawkins et al. 2008). HSP90 contains N-terminal ATP binding domain, substrate interacting middle domain, and C-terminal dimerization domain (Jackson 2013). HSP90 regulates myosin thick filament formation and muscle myofibrilogenesis by ATP binding to the N-terminal ATP-binding domain (Hawkins et al. 2008). HSP90 binds protein kinases, steroid receptors, intermediate filaments, microtubules, and actin microfilaments in a specific manner. HSP90 functions in glucocorticoid receptor. HSP40 and HSP70 are cochaperones with the HSP90s. HSP100 is located in the cytoplasm and cochaperones with HSP40, HSP70, and HSP90. HSP100 plays a role in refolding the aggregate. HSP110 is found in the cytosol and nucleus (Dimauro et al. 2016). HSP110 and HSP70 super family commonly have the presence of a loop structure and HSP110 helps immune response (Zuo et al. 2016). HSP110 is also in working with HSP70 or GRP78 to fold proteins and counter stress for cell survival (Gething and Sambrook 1992; Hartl 1996).

8.4 Factors Regulating Heat Shock Proteins

8.4.1 Generalized Stress Biomarkers in Environmental Toxicology

Biomarkers provide direct evidence of stress or potential stress. One major application is the use of stress proteins as molecular biomarkers for environmental monitoring, for both estimating environmental exposures to toxic chemicals, and detecting damage resulting from such exposures. These biomarkers rely on biochemical, histological, morphological, and physiological changes in whole organisms; however, changes at the cellular and molecular levels of organization, especially in nucleic acids and proteins, are increasingly being used to supplement these more traditional biomarkers (Ryan and Hightower 1996). Industrial and anthropogenic sources, such as urban runoff, sewage treatment plants, domestic dumping enreaches heavy metals (chromium, Cr; cadmium,Cd; and lead, Pb) in the aquatic systems (Heath 1987; Pinto et al. 2003; Sampaio et al. 2008). Downs et al. (2006) used protein-based biomarkers including HSP, cytochrome p450s, heme oxygenase, SOD, catalase and DNA-repair enzymes that were indicative of exposure to fuel oil. Woo et al. (2009) reported that exposure to Cr and Cd heavy metals for 24 h would change the transcriptional levels of eight genes related to oxidative stress in the liver of Japanese medaka. Whereas Li et al. (2014) demonstrated that the exposure to 0.5 and 2.5 mg/L of Cd for 4 days caused significant changes in the expression profiles of genes related to the hypothalamic- pituitary-thyroid (HPT) axis in Chinese rare minnow (*Gobiocypris rarus*), which effects in significant decrease of the thyroid hormone (TH) levels in the whole-body of fish.

Aquatic animals can be highly toxic to arsenic at the higher concentration than a permissible amount (Kim and Kang 2015). Arsenite (As^{3+}) is mainly existent in the aquatic environment, inorganic arsenic and arsenate (As^{5+}), which are interconverted via redox and methylation reactions (Bears et al. 2006). Most of the aquatic organisms have biotransformation mechanisms from arsenic to less toxic forms, because the organisms cannot keep away the arsenic exposure. Sulfhydryl groups of proteins induced the arsenite (As^{3+}) toxicity, whereas phosphorylation reactions involved in the arsenate (As^{5+}) toxicity (Hughes 2002). HSP70 considered as a reliable biomarker for the metal and metalloid exposure induced by the general stressor factors has a function to maintain protein integrity for the exposure to the toxic substances (Rajeshkumar and Munuswamy 2011). Boone and Vijayan (2002) observed a notable HSP 70 gene induction in rain-bow trout, *Oncorhynchus mykiss* exposed to copper, cadmium, and arsenic. The waterborne arsenic exposure to *S. schlegelii* caused a significant increase in HSP 70 gene, which indicates the water borne arsenic exposure affected the *S. schlegelii* as a substantial stress.

MT has known roles in routine physiological processes involving essential metals and in detoxifying non-essential metals such as cadmium (Amiard et al. 2006). Different MT isoforms can also be responsive to oxidative stress (Hook et al. 2006). Diniz et al. (2007) reported a significant increase in the MT of Asiatic clam, *Corbicula fluminea* by the trivalent arsenic exposure. Schlenk et al. (1997) also demonstrated a significant expression in the MT gene of channel catfish, *Ictalurus punctatus* exposed to arsenic. Whereas Kim and Kang (2016) have been reported, the waterborne arsenic exposure *to S. schlegelii* induced a significant increase in the MT gene expression, which may be induced by its protective role of MT to protect them from the cell damage by binding the metalloidions.

Fish are an excellent vertebrate model to investigate the physiology, function and regulation of HSP, because they are exposed to thermal and other stressors in their natural environment. Development and aging, stress physiology and endocrinology, immunology, environmental physiology, stress tolerance and acclimationare are effected by the functions of HSP (Basu et al. 2003). Heat shock proteins have constitutive functions that are essential in protein metabolism in the unstressed cell (Morimoto 1993). To validate the use of the HSP response as an indicator of stressed states in fish there have been several efforts. For example, increased levels of various HSP have been measured in tissues of fish exposed to industrial effluents, polycyclic aromatic hydrocarbons (Vijayan et al. 1998), several metals such as copper, zinc and mercury (Williams et al. 1996), pesticides (Hassanein et al. 1999) and arsenite (Grosvik and Goksoy 1996).

Temperature, heavy metal exposure and bacterial infections affect the survival and growth of aquatic organisms and results in serious losses in aquaculture (Wang et al. 2007; Liu et al. 2010). The negative feedback mechanisms of HSP genes are activated when organisms are under stress and degenerative proteins are increased. First, HSP70-Stip1-HSP90 complexes are bound to degenerative proteins and resulting in the dissociation of heat shock transcription factors (HSFs) from complexes. Then, HSFs assemble into trimmers and accumulate in the nucleus. Next, the three HSFs bind to heat shock element (HSE) and initiate the transcription of HSP genes (Ming et al. 2010; Moromoto 1993). Several studies have indicated that HSP70 expression was increased significantly under various stresses (Han et al. 2017; Giri et al. 2014). Therefore, some researchers have suggested that HSP70s could be used as a new biomarker for monitoring environmental stress (Han et al. 2017).

8.4.2 Heat Shock Protein Involved in the Immune Response in Fish

HSP play key roles in response to potential stress conditions (Lindquist and Craig 1988). HSP can be grouped into several families based on their apparent molecular masses: HSP110, HSP90, HSP70, HSP60 and low molecular weight HSP (Basu

et al. 2002). Aquatic animals generally face diverse types of environmental stress, such as thermal shock, virus, bacteria, and oxygen levels (Li et al. 2009). To adapt to these streesful environments, aquatic animals must develop an effective and helpful system. It has been demonstrated that HSP is responsible for protecting animals from stressful conditions. The report about the response of HSP90 gene at functional level in teleost remains deficient.

Fish are exposed to many kinds of stressors such as microbial infection, toxic exposure, traumatic damage, radiation, or nutritional deficiency. Therefore, the modulation of HSP genes is more apparent in fish and can be studied as molecular biomarkers of stress (Kayhan and Duman 2010). Microbial infection is the most damaging stressor that can modulate the regular physiological activity of fish (Sung and Mac Rae 2011). Both in the host and in parasites stress gene expression is regulated by infection (Henderson et al. 2006). Molecular chaperones that have dual roles as pathogen associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs), are probably the only protein involved in infections. Certain bacterial proteins were found to bind CD14 and TLR2/4 on monocytes and vascular endothelial cells of the host (Henderson 2003). Again, chaperones provides cell surface receptors for lipopolysaccharides (LPS) (Triantafilou et al. 2001). A host can recognize its own molecular chaperones and those of the invading bacteria. During bacterial infection HSP gene expression (HSP90 and HSP70) was previously studied in fish (Sung and Mac Rae 2011). It was found that HSP90 is involved in bacterial immunity, and the expressin is increased during viral infections in fish (Lee et al. 1996; Chen et al. 2010).

HSP70 gene was the most widely studied heat shock protein in fish during infections. The full lengths of hsp genes and corresponding immune responses have been identified in several fish species, which includes the responses of *Sparus sarba* to *Vibrio alginolyticus*, *Miichthys miiuy* to *Vibrio anguillarum*, *Ctenopharyngodon idella* to lipopolysaccharide, and *Anguilla marmorata* to *Aeromonas hydrophila* infection (Wei et al. 2013; Liang et al. 2016). HSP70 expression is increased in liver and kidney tissues of Coho salmon, *Oncorhynchus kisutch* during *Renibacterium salmoninarum* infection up to 63 days post-exposure (Forsyth et al. 1997) which is the first report to explain the influence of heat shock protein genes in fish in response to pathogens. The induction was not limited to fish, but higher expression was also reported in infected crabs, clams, and shrimps as well during Gram-negative bacterial exposure (Cui et al. 2010; Rungrassamee et al. 2010). An induction of a 90 kDa protein happened when chinook salmon embryo cell line (CHSE-214) was exposed to heat shock. This protein was also induced when haematopoietic necrosis virus (IHNV) infects CHSE-214 cell line (Lee et al. 1996).

8.4.3 HSP Gene Involved in Immune Response to Pathogenic Challenges

Pathogens may be regarded as naturally occurring biological stressors, represent one of the most important challenges an organism may encounter. The correlations of increased susceptibility to disease during stress in fish species have been reported (Mock and Peters 1990). However, the mechanisms are poorly understood, and few studies have described the progression of fish diseases from a physiological perspective. Vibriosis is one of the most prevalent fish diseases caused by bacteria belonging to the genus Vibrio. Vibriosis caused by the small, gram-negative, motile bacterium *Vibrio anguillarum.* Vibriosis occurs in cultured and wild marine fish in salt or brackish water, particularly in shallow waters during late summer. There is evidence that *V. anguillarum* is normally present in the intestinal microflora and food of cultured and wild healthy fish. The temperature and quality of the water, the virulence of the *V. anguillarum* strain and stress on the fish are important elements influencing the onset of disease outbreaks. Clinical signs and pathology of the disease include skin ulcers or a septicemia characterized by erythema, hemorrhages, and anemia; red, necrotic, boil-like lesions in the musculature; erythema of the bases of the fins and around the mouth; and the absence of a leukocytic response (Hendrikson and Zenoble 1983). It can rapidly invade different tissues of eels to disrupt the expression of immune-relevant enzymes (Guo et al. 2014; Feng et al. 2014).

The heat shock responses are well characterized in many model organisms such as *Drosophila melanogaster*, mouse and *Arabidopsis*. However, the responses can vary from organisms to organisms (Feder and Hofmann 1999; Cotto and Morimoto 1999). In black tiger shrimp (*P. monodon*), HSP have previously been characterized by identifying full-length cDNA sequences of HSP21, HSP70 and HSP90 (Huang et al. 2008; Liu et al. 2004; Jiang et al. 2009). The HSP21 transcript has been shown to be heat inducible in pleopods and was down-regulated during the infection by white spot syndrome virus (WSSV) (Huang et al. 2008). The HSP70 was inducible in haemocytes under heat shock conditions and the increase in the HSP70 expression was correlated with the reduction of Gill-Associated Virus (GAV) replication (Vega et al. 2006). Similarly, HSP90 was heat inducible in brain, stomach and heart and may play a role in ovary maturation (Jiang et al. 2009).

The expression profiles of HSP21, HSP70 and HSP90 in gill of *P. monodon* exposed to *V. harveyi* revealed the involvement of the HSP genes in the immune response of *P. monodon* (Jiravanichpaisal et al. 2006). Upon pathogen invasion, *P. monodon* triggers innate immune responses as their defense mechanisms and phagocytosis mainly takes place in hemocytes. The release of reactive oxygen species (ROS) during phagocytosis process helps in killing pathogenic bacteria. However, accumulation of ROS is harmful to host cell proteins, resulting in a similar protein damage to heat shock stress (Imlay and Linn 1988). Since, HSP have been well characterized as molecular chaperones, which assist in protein folding and repairing along with the significant induction of the HSP70 and HSP90 transcripts in

hemocytes under heat shock stress also suggests for possible involvement of HSP in shrimp immune response.

HSP works as a 'dangerous signal' to protect the immune system and the immune cells involved in the protection of cytoplasmic components (Ramaglia et al. 2004). In the immune system, it can be used to identify the important antigens for two reasons: firstly, the mRNA expression of *HSP* in most organisms revealed an apparent increase in the process of immune response when pathogens are engulfed by macrophages, in order to protect the organism. Secondly, HSP is highly conserved, and the immune system can easily identify these highly conservative molecules. During bacterial invasion, organism may release certain cell toxins, and promote intracellular cytokine synthesis and secretion caused by the variation of protein or polypeptide chain fragments (Han et al. 2017). These abnormal proteins can be induced by *HSP* genes in cells with high expression efficiency (Clark and Peck 2009). Recent studies indicated that HSC70 also plays important role in organism in response to environmental stressors, such as heat shock, heavy metal exposure and bacterial infection (Ming et al. 2010; Li et al. 2014). Multiple HSP70 family members such as HSP 70a1 (HSPa1), heat shock 70 kDa protein 2-like (HSPa2), heat shock 70 kDa protein 4-like (HSPa4), heat shock 70 kDa protein, heat shock cognate 70 (HSPa8), and heat shock 70 kDa protein 14-like (HSPa14) have been identified fish (He et al. 2013; Ao et al. 2015; Han et al. 2017). Members of the HSP70 family are involved in embryonic and gonadal development, as well as spermatogenesis, and protection against environmental stress. HSPa2 plays important roles during meiosis and post-meiosis (Dix et al. 1996; Govin et al. 2006; Hatfield and Lovas 2012). In addition, HSPa8 and HSPa4 are involved in embryogenesis (Han et al. 2017).

It is necessary to investigate the functions and expression characteristics of HSP under various stresses because of the important roles of HSP in disease and stress resistance. Cytoplasmic HSP70 family consists of HSP70 (inducible genes) and HSC70 (constitutive genes) subfamilies with many members in each (Li et al. 2015). He et al. (2013) have shown that there are atleast 14 HSP70 genes in swamp eel. In fish, HSP70 expression has been studied in in all major tissues, such as blood, kidney, spleen, and liver (Rajeshkumar et al. 2013; Qi et al. 2014). In *Schizothorax prenanti*, Sp-HSP70 and Sp-HSC70 were constitutively expressed in different organs, with the highest expression in the head kidney and blood, respectively (Liu et al. 2004). In *Sebastes schlegeli*, HSP70 mRNA is strongly expressed in head kidney (Mu et al. 2013). Han et al. (2017) investgated the expression analysis of five heat shock protein 70 (HSP70) family members in *Lateolabrax maculates*. They have reported that the five members of the HSP70 family are ubiquitously expressed in all examined tissues. However, these results are similar to the findings from other species, such as *Paphia undulata* (Wu et al. 2014), *S. schlegeli* (Mu et al. 2013), and *Labeo rohita* (Giri et al. 2014). HSP70 family members have tissue special function. For example, HSPa1 proteins can be released from cells and act as messengers to play an important role in the immune system (He et al. 2013). HSC70 is involved in vitellogenin uptake and testicular development (He et al. 2013). Some researchers

suggested that HSP expressed in spermatogenesis to provide for their specialized needs and serve as molecular chaperones for protein synthesis, folding, and transport (Rosario et al. 1992). The mechanism of HSP70s in spermatogenesis is needed to be studied further.

8.4.4 Heat Shock Protein in Cellular Response to Ultraviolet Radiation

Decline of ozone concentration in the stratosphere has raised concerns about increased solar UV radiation on the earth's surface including aquatic environment. UV carries more energy per photon than any other wavelength reaching the earth. Such high energy photon can potentially damage many biological molecules (Groff et al. 2010). Of the three type of incoming UV radiation, UV-B (280–320 nm) remained stressful to the terrestrial as well as aquatic plants and animals including human (Sucre et al. 2012). Exposition of planktonic, freshwater fish eggs and their larvae to UV-B radiation results in increased mortality and may lead to poorer recruitment to adult populations (Singh et al. 2013). The ultraviolet radiation is associated with the production of ROS including free radicals from oxygen and other oxygen-derived compounds (Hader et al. 2007; Sucre et al. 2012). These compounds are prooxidants capable of generating free radicals during its metabolism and producing biological effects lasting from minutes to years. Excessive exposure to UV radiation may overload the protection capabilities of organisms (Val et al. 2004).

Damage induced by UV radiation, can be at molecular level, organ, tissue and cellular leading to increased mortality of gametes and fish larvae (Hader et al. 2007; Dahms and Lee 2010). Prolonged exposure of fish to UV-B radiation often results in DNA damage that can pose a greater threat to organism's survival (Mitchell et al. 2004; Vehniainen et al. 2012). If DNA damage is not repaired, cells undergo complex enzymatic reactions expends high energy that might lead to apoptosis, necrosis or other forms of cell death (Sandrini et al. 2009). Some ROS-induced protein modifications can result in unfolding or alteration of protein structure, and some are essentially harmless events. Proteins are possibly the most immediate vehicle for inflicting oxidative damage on cells because they are often catalysts rather than stoichiometric mediators; hence, the effect of damage to one molecule is greater than stoichiometric (Dalle-Donne et al. 2003). Stress proteins are kind of sensitive markers of cell injury as these are associated with the immune response and autoimmune diseases (Roberts et al. 2010). Heat shock protein (HSP 70) is widely distributed group of HSP and its expression is markedly induced in response to environmental stresses, such as heat shock, UV and c-irradiation, and chemical exposure (Yamashita et al. 2010). It assists in the folding of nascent polypeptide chains that act as a molecular chaperone and mediate the repair and degradation of altered or denatured protein. The overall biology of oxidative protein modifications

remains complex and less understood. However, protein carbonylation is quite well characterized (Carrasco-Malio et al. 2014).

8.5 Heat Shock Protein Regulate Sexual Differential in Fish

Sex determination and differentiation in animals are greatly pliant, and vertebrates display different strategies. HSP are good candidates because of their thermal-sensitive expression (Bukau et al. 2000). Sex steroids could influence sex differentiation in many fish species (Kobayashi et al. 2011; Singh 2013), and research has proved that sex steroid levels can effect HSP expression (Romani and Russ 2013). Extensive researches on the molecular action of steroid hormone receptors indicate that steroid receptors appear to rely on HSP chaperone complexes for folding, regulation, and recycling (Didier 2006). Besides, some HSP are associated in gonad development and spermatogenesis. HSPa2 (also kown as HSP70.2) plays an important role during meiosis in mouse (Dix et al. 1996), and its absence results in infertility in male mice (Dix et al. 1996). Many HSP and related genes have sexually dimorphic expression and are involved in both metabolism and sex differentiation (Kohno et al. 2010; Boulangé-Lecomte et al. 2014). Temperature or sex steroid could influence sex differentiation in many species (Mork et al. 2014), and research has proved that sex steroid levels can effect HSP expression (Romani and Russ 2013).

8.6 Molecular Functions Including Stress Removal as They Are Most Important HSP

HSP have a critical role in the recovery of cells from stress and in cytoprotection, guarding cells from subsequent insults (Bukau and Horwich 1998). HSP interact with multiple key components of signaling pathways that regulate growth and development. The molecular relationships between HSP, various signaling proteins and partner proteins appear to be critical for the normal function of signal transduction pathways. The relative levels of these proteins may be important, as too little or too much HSP70 or 90 can result in aberrant growth control, developmental malformations and cell death. Thus, overexpression of HSP is an important means of cellular protection during physiological stress. HSP70 and 90 are the predominantly expressed stress-inducible proteins in eukaryotic cell.

HSP70 functions to refold or eliminate misfolded/denatured proteins playing critical role in many aspects of cellular signaling. HSP70 can function alone to inhibit apoptosis while cooperative interaction with their designated co-chaperone molecules is likely to enhance their anti-apoptotic activities (Beere et al. 2000). HSP90 is unique among molecular chaperones (Xu and Lindquist 1993). It is a

highly conserved, cytosolic, abundant protein of eukaryotic cells necessary for viability under all conditions and also aids in cell proliferation (Soti et al. 2005). HSP90 is activated when supporting various components of the cytoskeleton and steroid hormone receptors (Pearl and Prodromou 2000; Young et al. 2001). Heat shock protein synthesis is tightly regulated at the transcriptional level by heat shock transcription factors (HSF). The induction of HSP in response to stress is mediated largely through the transcriptional activation via HSF1 (Pirkkala et al. 2001). As metabolically active tissues vital to the maintenance of pregnancy, placental tissue is continuously in the stage of proliferation and apoptosis. HSP-like HSP90, key HSP involved in cell proliferation and HSP70, an important antiapoptotic protein were assessed along with the transcriptional HSP regulator HSF, in order to assess its role in preeclamptic endothelial cell. Simultaneously, an increase in the expression of HSF1, HSP70, and HSP90 was also observed. Previous reports suggest that HSF1, a nuclear transcription factor of HSP synthesis, is one of the target genes containing the hypoxia responsive element (Baird et al. 2006). The increased HSF1 would aid in increasing the expression of HSP70 and 90 which protects the cells from damaging stimuli. Both the proteins HSP70 and 90 have anti-apoptotic function (Pelham 1982; Orosz et al. 1996).

Scientific data suggest that HSP27 and other small heat shock proteins play role in development and aging. The HSP70 family is the most highly conserved and largest member of all the heat shock protein families. It presents in the sub-cellular compartments and primarily binds to target proteins to modulate protein folding and transport (Tavaria et al. 1996; Yokoyama et al. 2000). Under adverse environmental conditions, HSP70 improves expression levels and takes part in defense, repair or the detoxification machinery of the cell (Barnes et al. 2002; Jing et al. 2013). Many studies have shown that the transcription of HSP70 in some kinds of aquatic animals is simultaneously and differentially modulated upon exposure to environmental stressors (Deane and Woo 2006; Holm et al. 2008; Han et al. 2011; Jing et al. 2013).

The majority of studies on HSP in fish have been limited to *in vitro* examinations conducted in laboratory environments. Furthermore, most of these studies reported the induction of HSP families following exposure to stress. It is noteworthy that while many indicators of fish stress (e.g. plasma cortisol concentrations) are altered by handling and sampling procedures, Vijayan et al. (1998) demonstrated that handling stress does not alter levels of hepatic HSP70 in rainbow trout (*Oncorhynchus mykiss*).

Most of the studies have determined the impact of stressors on either oxidative or nitrative stress markers or HSP responses in fish *in vitro* by exposing them to a single anthropogenic stress under controlled conditions, but neglected to consider the cumulative effect of all stresses to which the organisms are subjected within their environment. However, a relatively little work on fish cells especially on whole organisms has been conducted. Under pollution-induced oxidative and nitrative stress condition, the liver mtHSP70 overexpression of Ennore fish sample was found to be responsible for the integrity of mitochondrial respiratory chain complexes and hence reduced free radical damage (Padmini et al. 2009; Padmini and Vijaya Geetha 2012). It is worty to mention that Mitochondria contain the set of

HSP70 that are essential for the maintenance of sufficient mitochondrial membrane potential necessary for efficient protein import (Moczko et al. 1995). Although the role of HSP in fish at the protein level has been investigated in many studies, very little is known about the genes that encode HSP in fish (Basu et al. 2002). The fish has some capacity to adapt themselves in response to stress in an effort to stabilize cellular function (Padmini and Vijaya Geetha 2012).

8.6.1 Heat Shock Protein Biomarkers in Heavy Metal Stress

Rapid industrialization and agricultural and anthropogenic activities during the recent decades have posed a serious threat to aquatic ecosystems. Heavy metals are hazardous pollutants that have a significant ecological impact and can modify the physical and chemical properties of water, thus affecting aquatic flora and fauna. Among the adverse effects, heavy metals result in mortality; alterations in haematological parameters, metabolism, reproduction, development; and immunodeficiency in fish (Jing et al. 2013; Giri et al. 2016). Cadmium (Cd^{2+}), lead (Pb^{2+}), and copper (Cu^{2+}) are heavy metals that frequently pollute the water environment. Cu exerts toxic effects on organisms once its concentration exceeds the supraoptimal level (Blanco-Penedo et al. 2006). Cd and Pb are nonessential metals that are toxic to organisms even in trace amounts (Pretto et al. 2010). These metals pose a threat to humans through the food chain (Chale 2002).

The HSP family proteins, especially, HSP70 and HSP90, are sensitive to environmental stressors, such as temperature and salinity (MiĆoviĆ et al. 2009), heavy metal (Giri et al. 2016), nutrient starvation (Han et al. 2011; Lauritano et al. 2015), ocean acidification (Harms et al. 2014; Moya et al. 2015), and chemicals produced by other marine organisms (Lauritano et al. 2016). Therefore, HSP70 and HSP90 have been used as biomarkers to monitor stressors in environmental toxicology (Gao et al. 2008; Zhang and Zhang 2012). Bivalve molluscs are sedentary and filter-feeding organisms that collect contaminants, such as heavy metal, pesticides, and hydrocarbons, in water. Therefore, these molluscs can be used to monitor aquatic environmental pollution (Gao et al. 2008; Manfrin et al. 2010; Ulloa et al. 2013; Liu et al. 2014). The Asian clam *Corbicula fluminea* is used as a biological indicator to monitor environmental changes in many fields and laboratories (Vasconcelos et al. 2009; Spann et al. 2011). Recent studies have identified several genes and response mechanisms related to pollution and stress (Rodius et al. 2005; Chen et al. 2013).

HSP70 and HSP90 are ubiquitously expressed with different expression levels under normal conditions. In a recent study, we demonstrated that Cd exposure led to the up-regulation of HSP47, HSP60, HSP70, HSP78, and HSP90 in head-kidneys and liver of *Labeo rohita*, indicating Cd-induced cellular stress (Giri et al. 2016). The RpHSP70 and RpHSP90 genes from *Ruditapes philippinarum* were ubiquitously expressed in tissues such as digestive gland, gill, adductor muscle and mantle (Liu et al. 2014). Similarly, PuHSC70 mRNA from *Paphia undulate* was expressed in all tested tissues (mantle, digestive gland, adductor muscle, gonad, gill, heart, and

hemocytes), and the highest expression levels were detected in the digestive gland (Wu et al. 2014). However, a different result was observed in *C. hongkongensis*, i.e., the highest chHSP70 expression level was detected in the muscle. The mRNA expression of HSP is usually induced by environmental factors, such as heavy metals, chemicals, and organic compounds; mRNA expression is more sensitive and earlier to respond to environmental stressors compared with traditional detection methods, such as mortality tests and growth inhibition (Feder and Hofmann 1999).

The expression of HSP90 at the transcriptional level greatly varies in different species under different heavy metal stressors. HSP90 expression from *Chlamys farreri* was induced by three heavy metals (Cd^{2+}, Cu^{2+}, and Pb^{2+}), and a dose-dependent expression was detected 10 days or 20 days after heavy metal exposure (Gao et al. 2008). The mRNA expression levels of ptHSP90–1 and ptHSP90–2 in the gills of *Portunus trituberculatus* were evidently increased by Cu^{2+} exposure (Zhang et al. 2009). The mRNA expression of HSP90α in the kidney of carp (*Cyprinus carpio*) increased under 10 mg/L Cd^{2+} exposure, peaked, and then gradually reduced (Hermesz et al. 2001). A number of researchers have criticized the use of HSP70 or HSP60 as biomarkers. Therefore, validation of stressor-specific risk assessment was considered through further research with larger groups of proteins (Mahmood et al. 2014). Mirkes et al. (1994) reported that the heat shock response, characterized by the synthesis and accumulation of HSP72, was not a general biomarker in rat embryos for chemical teratogens such as N-acetoxy- 2-acetylaminofluorene, $CdCl_2$, cyclophosphamide, sodium arsenite (AS), and sodium salicylate (SAL). Last two chemicals induced the synthesis and accumulation of HSP72, and both have different accumulation kinetics; otherwise, these chemicals caused embryotoxicity characterized by abnormal development and growth retardation. Overexpression of HSP72 after short-term exposure (2–6 hr) of pulmonary cell line (A549) to acute Cd concentrations (higher than 50 M) was considered an early biomarker for occupational exposure to Cd but long-term (1 month) chronic exposure *in vivo* made it doubtful because the expression of HSP72 decreased due to cellular adaptation to chronic Cd exposure (Croute et al. 2000; Mahmood et al. 2014). Similarly in juvenile rainbow trout exposed to Cd (1.5 g/L) and Zn (150 g/L) for 21 days, an adaptive response, to a lesser extent, in the liver was shown by an increase in antioxidant defenses (total glutathione, superoxide dismutase, and Trolox equivalent antioxidant capacity) without any impairment of GSH redox status or induction of HSP70 and HSP60 (Ait-Aissa et al. 2003). Although elevated temperature is the classic inducer of heat shock proteins, it is now clear that a variety of other stresses including pesticides, heavy metals, solvents and effluents can induce heat shock proteins (Stringham et al. 1992; Stringham and Candido 1994; Candido and Jones 1996; Mutwakil et al. 1997; Chowdhuri et al. 1999; Nazir et al. 2006; Gupta et al. 2007; Siddique et al. 2007, 2009; Singh et al. 2009). Under stress conditions, the newly synthesized stress proteins play an essential role in maintaining cellular homeostasis by assisting correct folding of nascent and stress accumulated misfolded proteins, preventing protein aggregation or promoting selective degradation of misfolded or denatured proteins (Schlesinger 1990; Morimoto 1993; Sikora and Grzesiuk 2007; Saluja and Dudeja 2008). The HSP can also affect cell survival by

interacting with various components of the programmed-cell death machinery, both upstream and downstream of the mitochondrial events (Garrido et al. 2001; Parcellier et al. 2003; Lanneau et al. 2008).

8.6.2 Heat Shock Protein Biomarkers in Cell Lines

When cells are exposed to a stressor, the rapid increase in HSP70 levels have been shown to protect the cells from the harmful effects of the stressor (Wang et al. 2007). Owing to its highly inducible nature of HSC70, this protein acts as a perfect mediator of cellular stress. The first stress response of cells is a general response characterized by an increase in stress proteins. Hence, HSP expression is commonly used as indicator of cellular stress in animals. The intracellular signal for induction of HSP is a sudden increase of stress induced abnormal polypeptides in the cytosol or nucleus (Sherman and Goldberg 2001). Moreover, few studies have assessed the cellular stress response in different cell types, with a relatively little work on fish cells especially on whole organisms (Iwama et al. 1999).

Cells have evolved different networks of cellular stress responses to adapt during environmental changes and survive combating wide variety of stress. The key members of this adaptive response include the antioxidant proteins thioredoxin (Dix et al. 1996; Nakamura 2005) and HSP (Calabrese et al. 2006). These proteins together form a powerful system in many intra and extra-cellular processes including protection against oxidative stress, antiapoptotic functions, regulation of the redox state of the extra-cellular environment and cell proliferation (Samali and Orrenius 1998; Calabrese et al. 2006). Among them, HSP90α is the most abundant, inducible molecular chaperone with essential roles in stress tolerance (Choi et al. 2008). It acts as a key component in the cellular multi-chaperone complexes actively involved in the regulation of the redox status of other proteins by assisting in the formation and breakage of disulphide bridges (Raina and Missiakas 1997; Rietsch and Beckwith 1998). It has also been documented that HSP90α is involved in the activation and maintenance of a wide range of regulatory and signaling proteins of cell proliferation, suggesting its critical role in cell growth and/or survival (Neckers 2007).

It has been demonstrated earlier that fish inhabiting contaminated estuary compared to uncontaminated extuary, experiences severe oxidative stress and overexpression of HSP like HSP70 and HSP90α under similar condition was in an effort to combat stress status and mount a protective response for survival (Padmini and Usha Rani 2008, 2009). Function of stress proteins and their role in cellular protection and survival also been demonstrated by other researchers (Basu et al. 2002; Cheng et al. 2006). However, these works were limited to different cell types with a relatively little work on fish cells. The phenomenon of the survival mechanisms adapted by a fish to resist against stress situation is still undeciphered in natural condition. Induction of antioxidant proteins like thioredoxin (Trx) and heat shock protein 90α (HSP90α) is a crucial step in the cellular response to oxidative stress

(Padmini and Rani 2010). HSP90α isoform is essential for maturation and activation of proteins. It plays appreciable roles in stress tolerance, protein folding and regulation of signal transduction pathways that control cell growth and survival (Parsell et al. 1993). Its role in the regulation of cell survival and death pathways via the modulation of the activity of a vast number of its client proteins is highly documented (Picard 2002; Sreedhar et al. 2004).

Padmini and Usha Rani (2009) studied the overexpression of HSP90α under oxidative stress condition in test fish hepatocytes. They found that upregulation of HSP90α being mediated via the transcriptional induction of HSP90α genes by HSF1. Jacquier-Sarlin and Polla (1996) have reported a model for the redox regulation of HSF activation and HSP synthesis during Trx expression; correlating positively Trx role on HSP induction and cooperative nature of these antioxidant proteins as cytoprotectors. Hence significant induction of both these proteins observed in test fish hepatocytes confers tolerance against stress status and appears to be intimately related with regulation of signal transduction processes that offer a cytoprotective role (Nakamura et al. 1997).

In a study, Padmini and Rani (2010) investigated the impact of environmental stress on Trx and HSP90α expressions in freshly isolated hepatocytes of *Mugil cephalus* living in either a contaminated or uncontaminated estuary. Modulation in the activities of signal transduction molecules like apoptosis signal-regulating kinase 1 (ASK1) and c-Jun NH2-terminal kinase ½ (JNK1/2) were investigated to understand their functional role under natural stressed condition. Test fish hepatocytes demonstrated significant upregulation in the levels of Trx and HSP90α and insignificant inductions in the expression pattern of ASK1 and JNK1/2 than control fish hepatocytes. Findings this provided direct evidence that Trx and HSP90α induction in fish hepatocytes under stress may aid cell survival by negatively regulating ASK1 expression and thereby functionally antagonizing the apoptotic role of JNK1/2 in natural aquatic systems (Padmini and Rani 2010).

8.7 Conclusions and Future Perspective

Fish can be considered as an ideal model organism to study the regulation and functional significance of heat shock proteins. The functional, ecological, and evolutionary genomics of heat shock proteins could be investigated in fish. The effect of short and long term thermal stress can be studied in fish (Kelly et al. 2000). Many fish species have external fertilization, and large manipulable eggs and embryos. Thus, heat shock protein expression and regulation can be studied at all life-history stages in fish. Studies into the functional genomics of heat shock proteins in fish will provide substantial insight into the physiological and ecological roles of these highly conserved proteins. The precise functions of proteins in the HSP70 family have not been completely deciphered. However, the high degree of conservation of these proteins across species, coupled with their importance in cell survival in various

conditions, suggests that these HSP are critical for both normal cellular function and survival after a stress. Therefore, one of the primary means to gain insight into HSP function in both in vitro and in vivo systems has been to assess their cellular responses after a stress-related induction. Similarly, target species have also been studied from an environmental ecology perspective, so that detailed information has been derived on HSP production in relation to environmental temperature and other eco-stressors (Roberts et al. 2010), indeed HSP expression level estimation has been advocated as a unique environmental monitoring tool (Sanders 1993). Iwama et al. (2004) indicated that background pathology of stocks being monitored and other variables influenced HSP levels also, which could confound the significance of HSP levels in terms of pollution level assessment. They considered that HSP played no role in the acclimation process but there was a clear correlation between resistance of cells that had been heat shocked or hardened and cellular HSP level. In this chapter, we have extensively discussed the ecotoxicological aspects of HSP synthesis and their potential as an environmental monitoring tool that have major impact on environmental physiology, ecology and toxicology. Feder and Hofmann (1999) suggested that future directions are required to: (a) resolve how heat shock protein genes, their regulation, and function have co-evolved in response to environmental change, and (b) how the action of heat shock proteins at the molecular level leads to whole organismal stress tolerance. One of the fundamental questions about the role of heat shock proteins is the functional relationship between the cellular stress response, the organismal stress response, and physiological processes at higher levels of biological organization. There are many possible applications of measuring the stress response in fishes, and other aquatic organisms. They range from being able to resolve the generalized stress response in our experimental animals, separate from treatment-specific effects, to the monitoring of the quality of the aquatic environment through the stressed states of the organisms that live there. However, these applications can only be developed if there is unequivocal evidence for a relationship between the stressed state of the animal and the cellular stress response. The evidence showing that increased levels of HSP induce tolerance of cells, tissues, and whole fish to subsequent stressors suggests that it may be possible to develop strategies to enhance tolerance to stressors by inducing the cellular stress response (Iwama et al. 1999). Heat shock proteins are collectively only one of the molecular mechanisms that animals utilize to tolerate stress, and these proteins can have pleiotropic effects, interacting with multiple systems in diverse ways. Thus, the cellular stress response has impacts on, and is influenced by, processes at all levels of biological organization. Functional and evolutionary genomics approaches will be critical for understanding the complex and integrative regulatory mechanisms that animals invoke in order to cope with changes in their natural environment.

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Chapter 9
The Role of Heat Shock Proteins in Response to Extracellular Stress in Aquatic Organisms

Li Lian Wong and Dinh Thinh Do

Abstract Heat shock proteins (HSP) are class of conserved and ubiquitous molecular chaperones present in all living organisms from primitive bacteria to humans. Numerous evidences accumulated last decades have proven multiple functions of HSP in aquatic organisms. Besides fundamentally respond to thermal stress, HSP are also stimulated by other extracellular stresses including salinity, pH, hypoxia, pollutants and pathogens. The induction of HSP towards multiple environmental stressors is to protect aquatic organisms. The mechanism and pathway involved in HSP induction are relatively complicated but are systematic to most cellular stressors. Given the vital functions of HSP in cellular protein protection and immunity defences, HSP-based vaccines and HSP-induced compounds have been developed and applied for the health management of aquatic organisms. Aquaculture species treated with these products have shown increased HSP productions, which have effectively, protect them against various stresses. Application of HSP based therapy in aquaculture practices proves as a promising approach in boosting aquaculture production and sustaining food security.

Keywords Aquaculture · Aquatic organisms · cDNA · Gene expression · Heat shock protein-based therapy · Stressors

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Abbreviations

HSC Heat shock cognate proteins
HSE Heat shock elements
HSF Heat shock factors
HSP Heat shock proteins
PAH Polycyclic aromatic hydrocarbon
PCB Polychlorinated biphenyl
ROS Species reactive oxygen
SHSP Small heat shock proteins

9.1 Introduction

Organisms in aquatic environment are confronted with multiple stressors. Abiotic stressors such as fluctuations in temperatures, salinity, oxygen and heavy metals may increase the susceptibility of aquatic organisms towards pathogenic infections (Jia et al. 2017). Stresses are able to alter the physiological processes and disturb cellular homeostasis in aquatic organisms, potentially influencing their growth, production and survival (Aleng et al. 2015). In response to environmental stressors, cell would produce a range of stress related proteins that predominantly comprised of heat shock proteins families (HSP) (Sanders 1993). HSP are ancient and conserved chaperones that can be found in all living organisms (Feder and Hofmann 1999). HSP respond to various stimuli, including both biotic and abiotic stressors (Cha et al. 2013).

HSP play essential roles in the folding, assembly, transport and degradation of proteins (Benarroch 2011). Under stress condition, HSP function as molecular chaperones to assist the correct folding of nascent proteins, prevent protein aggregation and refold partially denatured protein (Aleng et al. 2015). HSP are categorized into major families according to their molecular mass: HSP100, HSP90, HSP70, HSP60 and small HSP group (Table 9.1). The counterparts of many

Table 9.1 Major Heat Shock Protein (HSP) families and their functions in aquatic organisms (according to Roberts et al. 2010 and Sung et al. 2011).

Protein	Function
HSP100	Stress responses, protein disaggregation and facilitation of protein folding and destruction
HSP90	Essential for viability, promotion of protein folding, degradation and signaling, activity modulation for regulatory proteins
HSP70	Chaperone, nascent protein folding, secretion, protein secretion and import into organelles, thermotolerance
HSP60	Chaperonin, assembly of oligomeric proteins and folding of monomeric proteins, immune defence involvement
sHSP	Protection from stress, cytoskeleton stabilization, apoptosis inhibition

members of HSP families are referred to as heat shock cognate proteins (HSC). In the cell, while HSP are induced by stress, HSC is expressed constitutively under normal unstressed conditions (Roberts et al. 2010).

HSP have been shown to play an important role in the health of aquatic organisms related to the host response to various stresses. Based on this principle, HSP have been applied to fish health management to prevent infectious diseases and improve aquaculture production. HSP-based vaccine derived from pathogenic organisms is a promising approach to stimulate HSP synthesis in host (Josepriya et al. 2015). Non-lethal heat stress is applied to increase HSP production without causing any detrimental effects to the fish (Sung et al. 2014). Another approach, HSP-induced compounds are increasingly used in aquaculture. TEXOE® known as Pro-Tex® are commercially available and their efficiency in HSP stimulation to protect aquaculture species from various stressors have been proven (Roberts et al. 2010).

In this chapter, we described all the identified types of HSP in aquatic organisms and their responses to a variety of environmental stressors. The application of HSP in the health management of aquatic organisms is also presented. We have also highlighted the general pathway and mechanism undertaken by HSP in response to extracellular stresses in aquatic organisms.

9.1.1 Heat Shock Proteins in Aquatic Organisms

9.1.1.1 HSP100 Family

Like other HSP families, HSP100 is conserved, comprising both heat-inducible and constitutive members (Queitsch et al. 2000). In the cell, HSP100 is reported to work with other chaperones and proteases to control the quality and amounts of many proteins (Maurizi and Xia 2004). HSP100 plays an important role in promotion of proteolysis of specific cellular substrates and regulation of transcription (Schirmer et al. 1996). HSP100 family is significantly important in stress tolerance of bacteria and fungi and higher plants (Queitsch et al. 2000). Under stress condition, HSP100 is induced to maintain the functional integrity of key polypeptide and enable misfolded proteins to become refolded (Weber-Ban et al. 1999; Richter et al. 2010).

9.1.1.2 HSP90 Family

HSP90 is the most abundant cytosolic heat shock protein family, accounting for 1–2% of total proteins in a normal cell (Fu et al. 2011). This family shows multiple functions in protein folding, protein degradation and cell signalling (Gething and Sambrook 1992; Pratt and Toft 2003; Pearl and Prodromou 2006). As a well-studied family (Pu et al. 2016), HSP cDNA has been reported in a number of aquatic species such as molluscs *Haliotis tuberculate*, *Chlamys farreri*, *Haliotis asinina*, *Crassostrea*

gigas, *Argopecten irradians*, *Laternula elliptica*, *Crassostrea hongkongensis*, and *Haliotis diversicolor* (Farcy et al. 2007; Gao et al. 2007; Gunter and Degnan 2007; Choi et al. 2008; Gao et al. 2008; Kim et al. 2009; Fu et al. 2011; Huang et al. 2014), fishes *Cyprinus carpio*, *Ctenopharyngodon idella*, *Acipenser schrenckii*, *Miichthys miiuy*, *Ictalurus punctatus*, and *Schizothorax prenanti* (Hermesz et al. 2001; Wu et al. 2012; Wei et al. 2013; Xie et al. 2015; Pu et al. 2016), sea cucumber *Apostichopus japo* (Zhao et al. 2011) and shrimp *Fenneropenaeus chinensis*, and *Exopalaemon carinicauda* (Li et al. 2009; Li et al. 2012). The expression of HSP90 is regulated by a variety of environmental stressors, including temperature, benzo (a) pyrene, pH, cadmium, crowding, hypoxia, salinity and bacterial challenge (Pu et al. 2016). In addition to chemical and physical stressors, biological stressors such as pathogenic microorganisms also modulate HSP90 gene expression. Recent studies showed that HSP90 was expressed in various tissues in oyster *Crassostrea hongkongensis* and fish *Schizothorax prenanti* (Fu et al. 2011; Pu et al. 2016). Accordingly, HSP90 levels were induced following pathogenic challenge test with *Vibrio alginolyticus* for oyster (Fu et al. 2011) and *Streptococcus agalactiae* for fish (Pu et al. 2016). Given its responses to a wide range of environmental stressors, HSP90 is a potential biomarker to evaluate the quality of aquatic environments.

9.1.1.3 HSP70 Family

HSP70 family is considered the most important HSP proteins (Li et al. 2015) and also the most thoroughly studied among all characterized HSP families (Rhee et al. 2009). Among HSP, HSP70 represents one of the most conserved and has been widely reported (Gupta et al. 2010). In eukaryotes, there are two genes encoding for HSP70 protein family, an inducible, HSP70 gene and a cognate HSC70 (Deane and Woo 2006). While HSC70s are constitutively expressed in unstressed cells, in relation to developmental progress, HSP70s are reported be induced by various stressors such as heat, chemicals and microorganism infection (Dang et al. 2010; Han et al. 2017). In terms of ecotoxicology, HSP70 is suggested as a sensitive biomarker for a wide range of adverse stressors (Scheil et al. 2009). Genes encoding for HSP70 have been described in a variety of aquatic species, including fishes such as, *Carassius auratus*, *Megalobrama amblycephala*, *Tanichthys albonubes*, *Sebastes schlegeli*, and *Schizothorax prenanti* (Kondo and Watabe 2004; Ming et al. 2010; Jing et al. 2013; Mu et al. 2013; Li et al. 2015), molluscs *Mytilus galloprovincialis*, *Pinctada fucata*, and *Meretrix meretrix* (Cellura et al. 2006; Wang et al. 2009; Yue et al. 2011), crustaceans *Mirocaris fortunata* and *Rimicaris exoculata*, *Tigriopus japonicas*, *Portunus trituberculatus*, *Scylla serrata* and *Macrobrachium rosenbergii* (Ravaux et al. 2007; Rhee et al. 2009; Cui et al. 2010; Fu et al. 2013; Chaurasia et al. 2015). Meanwhile, HSC70 cDNA has been sequenced in fishes *Oncorhynchus mykiss*, *Oryzias latipes* and *O. celebensis*, *Danio rerio*, *Scophthalmus maximus*, and *Lateolabrax maculatus* (Zafarullah et al. 1992; Arai et al. 1995; Graser et al. 1996; Wang et al. 2013a, b; Han et al. 2017), crustaceans *Penaeus monodon*, *Litopenaeus vannamei*, *Metapenaeusensis*, *Macrobrachium nipponense*,

and *Eriocheir sinensis* (Chuang et al. 2007; Wu et al. 2008; Chan et al. 2014; Xiu et al. 2014; Li et al. 2016a, b) and echinoderm *Apostichopus japonicas* (Wang et al. 2013a, b). In aquatic organisms, HSP70 function is well documented compared to other HSP families. Besides normal cellular functions such as folding and translocation of proteins, HSP70 is known to play essential roles in remediating the effects of environmental stressors, including heat shock, salinity fluctuation, oxygen deprivation, chemical exposure, and bacterial infection (Yamashita et al. 2010; Qi et al. 2014). HSC70 and HSP70 appear to be expressed in different patterns in the cell. HSC70 is actively expressed in stress conditions and remains unchanged or mildly induced by stimuli, while HSP70 is highly stimulated by stress conditions (Han et al. 2017). Evidently, HSP70 was induced following acute stress, while HSC70 was induced following chronic stress in *Mytilus galloprovincialis* (Franzellitti and Fabbri 2005).

9.1.1.4 HSP60 Family

HSP60 is a highly conserved and multi-functional chaperone that plays important roles in polypeptide folding and protein translocation (Van der Vies et al. 1993). Numerous studies have revealed possible functions of HSP60 in normal cellular processes such as sperm cell differentiation and reproduction development (Xu and Qin 2012). Additionally, HSP60 is known to be key component in stress response (Shi et al. 2015a, b). Previous studies demonstrated that HSP60 is involved in immune defence against pathogen (Quintana and Cohen 2011). In *E. coli*, HSP60 and its counterpart HSP10 assemble to form a cage-like macromolecule that facilitates proper folding and assembly of proteins and corrects misfolded polypeptides (Gupta et al. 2010; Shi et al. 2015a, b). HSP60 gene has been reported in diverse aquatic organisms such as fishes *Ctenopharyngodon idella*, *Paramisgurnus dabryanus*, and *Siniperca chuatsi* (Xu et al. 2011; Li et al. 2014; Wang et al. 2017), crustaceans *Portunus trituberculatus*, *Scylla paramamosain*, and *Penaeus monodon* (Xu and Qin 2012; Yang et al. 2013; Shi et al. 2015a, b). Apart from normal physiological functions in the cell, HSP60 play an important role in responding to heat and salinity stress (Yang et al. 2013). Shi et al. (2015a, b) reported that a recombinant expression of HSP60/ HSP10 in ATPase and chaperone in tiger shrimp *Penaeus monodon* has demonstrated an effective way in protecting this shrimp species against cellular stress.

9.1.1.5 Small HSP Family

Small heat shock proteins (sHSP) are a diverse group of HSP family that is characterized by low molecular mass of 12–43 kDa (Zhang et al. 2015). sHSP include the most common but also the most poorly conserved family of molecular chaperones (Richter et al. 2010). Like the other heat shock proteins, sHSP function as molecular chaperones that prevent aggregation of target proteins and support the

refolding of denatured proteins (Bakthisaran et al. 2015). While some sHSP are constitutively expressed, most of them are strongly inducible (Gupta et al. 2010). In addition to multiple functions in normal cellular processes, sHSP are demonstrated to play important roles in stress tolerance. sHSP expression is induced by various stressors, including heat, salt, drugs, viral infection and oxidants (Arockiaraj et al. 2012). sHSP are present in all three kingdoms of life, from archaea and bacteria to plants and animals (Waters and Rioflorido 2007). Variety of sHSP cDNA have been isolated and characterized in a range of aquatic organisms as HSP21 from shrimp *Penaeus monodon* (Huang et al. 2008), HSP37 from shrimp *Macrobrachium rosenbergii* (Arockiaraj et al. 2012); HSP26 from sea cucumber *Apostichopus japonicus* (Zhao et al. 2011), HSP20 from molluscs *Haliotis discus discus* and *Meretrix meretrix* (Wan et al. 2012; Li et al. 2013), HSP40 from *Pinctada martensii* (Li et al. 2016a, b), HSP27 from fishes *Danio rerio*, *Oncorhynchus mykiss*, *Carassius* auratus, and *Larimichthys crocea* (Mao et al. 2005; Ojima 2007; Wang et al. 2007; Ojima and Oohara 2008; Yang et al. 2012) and HSP30 from fishes *Poeciliopsis lucida*, *Carassius auratus*, and *Fundulus heteroclitus* (Norris et al. 1997; Kondo et al. 2004; Healy et al. 2010).

Unlike human and mammalian, information and studies related to sHSP are lacking for aquatic organisms. Previous studies indicated that sHSP gene expression was regulated by thermal, low salinity and bacterial challenges in pearl oyster (Li et al. 2016a, b); pollutants in *Meretrix meretrix* (Li et al. 2013); water temperature in brine shrimp *Artemia franciscana* and sea cucumber *Apostichopus japonicus* (Crack et al. 2002; Zhao et al. 2011); bacterial and viral infection in shrimps *Penaeus monodon* (Wanilada et al. 2010) and *Macrobrachium rosenbergii* (Huang et al. 2008; Arockiaraj et al. 2012). Huang et al. (2008) reported ubiquitous expression of HSP21 in various organs of *Penaeus monodon*. However, levels of HSP21 mRNA expression was down-regulated after white spot syndrome virus infection. This illustrates that gene regulation of HSP21 was critically controlled by white spot syndrome virus. In contrast, expression of HSP37 gene in *M. rosenbergii* (Arockiaraj et al. 2012) was up-regulated following infectious hypodermal and hematopoietic necrosis challenge test. It indicates potential involvement of HSP37 in the immune responses against viral challenge in *M. rosenbergii*.

9.1.2 Environmental Stressors and Responses of Heat Shock Proteins (HSP) in Aquatic Organisms

Increased pollutants from agricultural and industrial activities are posing severe threats towards the aquatic environment (Thinh et al. 2016; Birnie-Gauvin et al. 2017). In addition to physical and chemical pollution, rapid development of aquaculture industries increases global transmission of infectious disease to aquatic organisms. Under stressed conditions, aquatic organisms are able to enhance HSP production to prevent harmful effects. In this section, we will discuss different types of stressors found in the aquatic environments.

9.1.2.1 Temperature

Temperature is an abiotic factor that significantly influences growth, reproduction and survival of aquatic organisms (Mahanty et al. 2017). Despite its important role, temperature may acts as one of the common stressors that potentially intervene with the physiological mechanisms of aquatic organisms. Global climate change is predicted to increase water temperature. According to IPCC Report (2013), water temperature will increase by 2 °C at the end of the twenty-first century. In addition to increased temperature due to climate change, cold shock can naturally occur in natural events such as thermocline temperature variation, rapid changes in solar heat, abnormal water movements, rapid precipitation and rapid changes in seasonal temperatures (Wu et al. 2015). Water temperature fluctuation is reported as one of the most important stressors that affect the biological and physiological progresses at cellular and molecular levels (Chaurasia et al. 2015). Evidence accumulated over the past decades demonstrated that cells, tissues and organisms exposed to sub-lethal heat stress resulted in the production of HSP (Sung et al. 2011).

HSP expression has been known to improve thermotolerance in various aquatic species. The sublethal and lethal temperatures for the juveniles of sea cucumber *Apostichopus japonicus* were determined as 30 °C and 34 °C, respectively (Dong and Dong 2008). These juveniles challenged with sublethal temperature showed increase in HSP70 expression in the beginning, then decrease over time. Pre-exposure to sublethal heat shock (30 °C) for 2 h could increase the survival rates of the sea cucumbers when they were exposed to lethal temperature (34 °C). HSP production was shown to increased in many heat-exposed crustaceans with time-dependent manners (Liu et al. 2004; Selvakumar and Geraldine 2005; De la Vega et al. 2006; Sung et al. 2007). When Pacific white shrimp *Litopenaeus vannamei* exposed to thermal stress, all four heat shock proteins (HSP60, HSP70, HSC70 and HSP90) were induced, but HSP70 was the most sensitive protein among them to temperature fluctuations (Qian et al. 2012). There are numerous available evidences, which show that stimulation of different HSP families in fish under thermal stress (Lund et al. 2002; Lund and Tufts 2003; Rendell et al. 2006; Marvin et al. 2008; Werner et al. 2007; Wu et al. 2012). Previous extensive reviews have indicated important roles of HSP in increased thermotolerance in fish (Basu et al. 2002; Iwama et al. 2004; Sung et al. 2011). Recently, transcriptome studies have been conducted to evaluate the responses of multiple genes to temperature stress, including HSP. Accordingly, expression of HSP70 in grass carp *Ctenopharyngodon idellus* was found to be enhanced by heat stress (Yang et al. 2016).

In addition to heat shock, cold shock has also been reported to alter HSP expression in aquatic organisms. The expression of HSP70, HSP90–1 and HSP90 in swimming crab *Portunus trituberculatus* increased with decreasing temperature, reached the maximum levels at 6 °C and then dropped markedly at 3 °C (Meng et al. 2014). These results suggested that HSP in swimming crabs are enhanced to protect their cell from damage at low temperatures. However, cold stress beyond withstanding level could suppress the protective mechanisms, resulting in cellular damages. For molluscs, decrease in temperature from 20 °C to 3 °C for 3 hours led to increased

production of HSP70 and HSP40 in bay scallops *Placopecten magellanicus* (Brun et al. 2008). Interestingly, the response of HSP40 to cold shock was more rapid than for HSP70 in these scallops. Previous studies demonstrated that HSP synthesis in fish is enhanced by low temperature (Ju et al. 2002; Weber and Bosworth 2005; Shi et al. 2015a, b). It is evident that cold stress increased HSP70 mRNA both in liver and muscle of Nile tilapia *Oreochromis niloticus* (Shi et al. 2015a, b). The results suggested that up-regulation of HSP70 as the main response towards cold stress in tilapia has protected the fish from excessive damage. Similar HSP expression patterns have also been observed in our current study on *Tor tambroides* when thermal stress was employed on the fish (data not shown).

9.1.2.2 Hypoxia

Oxygen is a fundamental element that is essential for the existence of most living organisms. Different from oxygen concentration in the atmosphere, oxygen concentration in water is influenced markedly by temporal and spatial variations (Mohindra et al. 2015). Hypoxia occurs in aquatic systems when dissolved oxygen reaches to relatively low levels where it becomes detrimental to aquatic organisms (Vaquer-Sunyer and Duarte 2008). Although hypoxia can be caused by a number of natural factors, it is mainly caused by human activities. Increased anthropogenic nutrient loading into aquatic environment has resulted in eutrophication. In turn, eutrophication encourages phytoplankton blooms and subsequently hypoxia. This external stressor can negatively affect the behavior, survival, growth and reproduction of aquatic organisms (Thomas and Rahman 2009). Hypoxia forces aquatic organisms to develop a range of behavioural and physiological mechanisms in minimizing its potential detrimental effects. One of the important defence mechanisms is to increase the expression of heat shock proteins (Mohindra et al. 2015). HSP90 from Chinese shrimp *Fenneropenaeus chinensis* and HSP70 from oriental river prawn *Macrobrachium nipponense* were reported to be inducible by hypoxia (Li et al. 2009; Sun et al. 2016). In fish, levels of HSP70 and HSP90 indicated a remarkable increase after Amur sturgeon *Acipenser schrenckii* and Indian catfish *Clarias batrachus* were challenged with extreme low oxygen level (Ni et al. 2014; Mohindra et al. 2015).

9.1.2.3 Salinity

Salinity has important physiological effects on aquatic organisms. Species live in estuary regions can adapt to large fluctuations in salinity levels. Anadromous fish species undergo large salinity changes due to their life history patterns, which involve migration between ocean and river. However, salinity is a limiting factor that determines the survival and distribution of many aquatic organisms (Peng et al. 2015). Salinity fluctuation can alter osmotic pressure of coelomic fluid in aquatic organisms, resulting in osmotic stress. As a consequence, osmotic stress damages

proteins resulting in the disruption of protein synthesis (Meng et al. 2011). Large-scale mortality occurs in most marine invertebrates such as molluscs and echinoderms when the salinity level is reduced by more than 20% (Zhao et al. 2012). This may bring huge economic loss to aquaculture sectors, especially during the rainy season. Salinity alteration demands physiological and behavioural adaptations such as osmoregulation from aquatic organisms. To cope with this osmotic stress, aquatic organisms are forced to induce HSP synthesis. Previous studies demonstrated that low salinity could cause osmotic stresses and induce the up-regulation of HSP70 in sea cucumber *Apostichopus japonicusm* (Dong et al. 2008; Meng et al. 2011). Xu and Qin (2012) detected that HSP60 expression was significantly altered when *Portunus trituberculatus* exposed to different salinity levels. The results indicated the importance of HSP60 in regulation of salinity stress in *P. trituberculatus*. For fish, exposure to salinity fluctuation has also led to increased expression of various HSP (Deane and Woo 2011), indicating the significant role of these proteins in responses to osmotic stress.

9.1.2.4 Heavy Metal

Heavy metal contamination in aquatic ecosystems has received increased worldwide attention (Mansour and Sidky 2002). While essential metals, such as iron, copper and zinc produce toxic effects to aquatic organisms when present at high concentrations, non-essential metals such as cadmium are toxic, even at low levels (Subotić et al. 2013). To limit adverse effects of heavy metals, aquatic organisms have evolved a range of mechanisms which permit metal regulation in their body, including production of heat shock proteins. To date, a number of laboratory and field studies have been performed to evaluate the effects of heavy metals on HSP70 expression in fish. HSP70 expression is reported to be induced by heavy metals such as copper, zinc, mercury, nickel, cadmium, lead and arsenic (Sanders et al. 1995; Williams et al. 1996; Duffy et al. 1999; Boone and Vijayan 2002; Ali et al. 2003; Deane and Woo 2006; Fulladosa et al. 2006; Padmini and Rani 2009; Rajeshkumar et al. 2013). Boone and Vijayan (2002) exposed rainbow trout hepatocytes to $CuSO_4$, $CdCl_2$ and $NaAsO_2$ to investigate the effects of heavy metal on HSC70 and HSP70 expression. Their results indicated that while HSP70 level was induced, HSC70 level showed no change over the treatment. Fulladosa et al. (2006) collected blood cells from silver sea bream (*Sparus sarba*), and treated them with different sublethal concentrations of cadmium, lead and chromium. HSP70 was found to be remarkably over-expressed at a very low metal concentration of 0.1 M (Fulladosa et al. 2006). Rajeshkumar et al. (2013) estimated HSP70 expression in milk fish (*Chanos chanos*) at sites polluted with Cu, Pb, Zn, Cd, Mn, Fe. Compared to less contaminated sites there was an increased level of HSP70 in fish tissues from contaminated sites. HSP70 expression in different tissues showed that the expression intensity was high in fish sampled from polluted sites. They concluded that HSP70 is a useful biomarker for oxidative stress induced by heavy metal.

In addition to HSP70, other HSP in aquatic organisms are also documented to be stimulated by heavy metals. HSP60 in black tiger shrimp *Penaeus monodon* was significantly induced by cadmium, zinc and copper (Shi et al. 2015a, b). Similarly, diet containing high levels of zinc stimulated production of HSP26, HSP70 and HSP90 in abalone, *Haliotis discus hannai* (Wu et al. 2011). In fish, the expression of multiple HSP in *Labeo rohita* has been reported to be triggered by arsenic and cadmium. Arsenic exposure led to up-regulation of HSP47, HSP60, HSP70, HSP71, HSP78, and HSP90 while cadmium exposure resulted in up-regulation of HSP47, HSP60, HSP70, HSP78, and HSP90 in *Labeo rohita* (Banerjee et al. 2015; Giri et al. 2016). Taken together, aquatic organisms respond towards detrimental effects of heavy metal stress by the induction of HSP synthesis to prevent cellular and protein damages.

9.1.2.5 pH

Ocean acidification and reduction of pH level is associated with the rising atmospheric carbon dioxide CO_2 from human activities (Melzner et al. 2013). Low pH level may change water chemistry and increase dissolved heavy metals such as Fe, Mn, Zn, Al, Cr, Ni. Reduction of pH level in aquatic environment may influence intracellular pH, membrane function, energy partitioning, enzymatic activities and calcification rates in aquatic species (Berge et al. 2010). Aquatic organisms which undergo stress caused by low pH levels have shown lower rates of growth, reproduction, or survival (Pespeni et al. 2013). pH decline is known to have negative impacts on aquaculture species. It is reported that low pH resulted in apoptosis in shrimp *Fenneropenaeus chinensis* (Wang et al. 2011). Previous studies demonstrated that changes in water pH can increase intracellular species reactive oxygen (ROS), resulting in modulation of heat shock protein expression (Qian et al. 2012). HSP90 gene expression in hepatopancreas of *Exopalaemon carinicauda* showed upward trends after pH exposures (Li et al. 2012). Upon reaching the peak, HSP levels decreased gradually to the normal level at the end of the experiment (24 h). In another study, Qian et al. (2012) found that under acute pH challenge, mRNA expression of HSP60, HSP70, HSC 70 and HSP90 in shrimp *Litopenaeus vannamei* was observed to be time and pH-dependent, with HSP60 being the HSP with the strongest induction. The expression levels of HSP60 mRNA in gill and hepatopancreas of *Penaeus monodon* were up-regulated under pH challenge (Shi et al. 2015a, b).

9.1.2.6 Organic Chemicals

Anthropogenic activities have resulted in the discharge of a huge number of organic chemicals such as petroleum hydrocarbons, halogenated and nitroaromatic compounds, phthalate esters, solvents and pesticides to aquatic environments (Megharaj et al. 2011). Many organic pollutants found in aquatic environment have

accumulated in organisms, which have posed adverse effects on the aquatic ecosystem and human health. Persistence of organic chemicals in the organisms, water body and sediments depends mainly on their chemical and physical characteristics (Perelo 2010; Abdel-Shafy and Mansour 2016). Organic chemicals with complex structure, more halogenated and hydrophobic contents tend to accumulate in sediments (Perelo 2010). Ecotoxicological research on organic pollution has caught a lot of attention due to potential threats of these pollutants towards the aquatic biota (Collin et al. 2010; Xing et al. 2015). One aspect of these research is to determine the role of heat shock protein responses in aquatic organisms towards organic chemicals. Heat shock proteins are reported to be regulated by different organic chemicals such as pesticides, herbicides, polychlorinated biphenyl (PCB) and polycyclic aromatic hydrocarbon (PAH).

Deltamethrin is a synthetic chemical with strong pesticidal properties. It is reported that deltamethrin induced the expression of HSP70 in rainbow trout *Oncorhynchus mykiss* (Ceyhun et al. 2010). Atrazine (ATR) and chlorpyrifos (CPF) are common pesticides used in agriculture around the world. These pesticides were documented to stimulate the expression of HSP60, HSP70 and HSP90 in common carp *Cyprinus carpio* (Xing et al. 2015). Asian paddle crabs, *Charybdis japonica* which was exposed to Endocrine Disrupting Chemicals, Bisphenol A (BPA) and 4-Nonylphenol (NP) showed increased production of HSP70 (Park and Kwak 2013). Apart from laboratory research, field investigation revealed that HSP70 induction was also reported in brown trout *Salmo trutta* f. *fario* and gammarids *Gammarus pulex* sampled from rivers polluted with PCBs, hazardous air pollutants (HAPs) and other pollutants (Triebskorn et al. 2002). In another study, HSP mRNA in bass *Epinephelus guaza* was found higher in area contaminated with PAHs compared to control area (Abdel-Gawad and Khalil 2013).

9.1.2.7 Biotic Stressors

Aquaculture production has been rapidly growing over the past decades to support global food security (FAO 2016). Aquaculture activities are associated with a variety of potentially harmful stressors to aquatic organisms, particularly pathogens. It is reported that periodical disease outbreaks in fish cultures are contributed by 54.9% bacterial pathogens, 22.6% viruses, 3.1% mycotic agents, and 19.4% parasitic agents (Dhar et al. 2014). Aquatic organisms combat against infectious diseases through the production of heat shock protein to protect proteins and cells from stress. The responses of HSP to pathogenic viruses and bacteria were reported in different organisms, including molluscs, crustaceans and fish. Syntheses of HSP60, HSP70 and HSP90 in freshwater prawn *Macrobrachium rosenbergii* were stimulated by the infection of virus (white spot syndrome virus and *M. rosenbergii* nodo virus) and bacteria (*Aeromonas hydrophilla* and *Vibrio harveyi*) (Chaurasia et al. 2015). Yang et al. (2013) detected that HSP60 expression in mud crab *Scylla paramamosain* was increased after the crab was infected by *Vibrio alginolyticus*. This challenge test has resulted in higher production of HSP90 mRNA from *Crassostrea hongkongensis*

(Fu et al. 2011). In fish, HSP60 synthesis from grass carp *Ctenopharyngodon idella* increased after the fish was challenged with bacterium *Aeromonas hydrophila* (Xu et al. 2011). In another study, HSP70 and HSP90 from *Larimichthys crocea* were significantly up-regulated by bacteria *V. alginolyticus* (He et al. 2016).

In addition to pathogenic microbes, most aquatic organisms serve as hosts to a range of parasites. Parasites may reduce their host's growth, resistance to other stressors and reproductive ability (Scholz 1999). Parasites are able to modulate biomarker responses in organisms, including some that are routinely employed in ecotoxicological studies, such as metallothionein, cytochrome P450, oxidative stress enzymes, and heat shock proteins (Marcogliese and Giamberini 2013). Expression of heat shock protein in fish is modulated by different parasites (Fazio et al. 2008; Frank et al. 2013; Thinh et al. 2016). These findings highlighted the prominent functions of HSP in the first line of stress defence against pathogens in aquatic organisms.

9.1.3 Mechanism and Pathways of HSP Induction in Response to Extracellular Stressors

Knowing the mode of action of HSP in response towards stressors in aquatic animals is far more limited than understanding their role and significance. In this section, we are only able to discuss the general mechanism and pathways of HSP for vertebrates and fish (catfish) in dealing with extracellular stress. In general, response mechanisms of an organism towards stressors involve three fundamental phases: i) alarm reaction, ii) resistance stage to achieve homeostasis, and iii) exhaustion stage when homeostasis is not achieved. These series of physiological and biochemical changes are termed "General Adaptation Syndrome" (GAS) which are not species specific nor stressor specific, but differ with regards of the totality of the response for each stressor. The response towards various stressors at cellular level is generally through up-regulation of genes, blood and tissue levels of a group collectively known as heat shock proteins (HSP) (Feder and Hofmann 1999).

The heat shock response was first described by Ritossa (1962) as an observed aggregation of "clouds" in polytene chromosomes in the salivary glands of *Drosophila* larvae exposed to heat stress or to chemical toxins. Although heat shock is a typical inducer of heat shock proteins, a variety of other stressors such as pesticides, heavy metals, solvents and effluents have been reported to induce HSP (Mutwakil et al. 1997; Nazir et al. 2006; Gupta et al. 2007; Siddique et al. 2009). Denatured proteins in stressed cells may either be degraded by a cellular protease or refolded by molecular chaperones (Wickner et al. 1999). Under stress conditions, HSP accumulates at high levels and remain elevated for an extended period in the stressed cells (Lindquist and Craig 1988). HSP play an essential role in maintaining cellular homeostasis by ensuring correct folding of nascent proteins, preventing

inappropriate protein aggregation, and stimulating selective proteolysis of misfolded or denatured proteins (Sikora and Grzesiuk 2007; Saluja and Dudeja 2008). The HSP determine cell survival via interaction with programmed-cell death machinery (Lanneau et al. 2008).

Besides their fundamental roles in the regulation of normal protein synthesis, HSP also function as chaperones for all cellular protein and lipid metabolic processes, particularly in stress-induced condition. HSP are not only key components of the early response to stressors, but are vital for defence mechanisms against neoplasia and pathogenic infections (Srivastava 2002). The transcription of HSP genes is mediated by the interaction of heat shock factors (HSFs) with heat shock elements (HSE), which is a series of pentameric units of the sequence 5′-nGAAn-3′ found in all HSP genes promoter regions (Pockley 2003). Generally, there are three to four HSFs in finfish and other vertebrates, in which HSF1, HSF2 and HSF4 are ubiquitously expressed (Scharf et al. 1998; Pirkkala et al. 2001). HSFs are regulated differently for each stimuli under normal or stress condition, and thus have different transcriptional responses (Akerfelt et al. 2007; Abane and Mezger 2010).

Among all these characterized HSFs, HSF1 is the primary transcription factor responsive to all forms of cellular stress and conditions that induce the heat-shock response (Sarge et al. 1994). HSF2 is generally regulated for development and differentiation process such as erythrocyte differentiation, embryonic development, and spermatogenesis (Rallu et al. 1997). Both HSF1 and HSF2 normally complement each other in response to cellular stress or even during normal cellular state (Östling et al. 2007). The expression of HSF4 is tissue-specific, and is predominantly present in lens and brain (Rupik et al. 2011).

When exposed to chemical or biological stressors, polar bonds weaken which expose the cell towards hydrophobic groups resulting in protein denaturation, protein aggregation and protein misfolding within the cell or cell surface (Wedler 1987). Following these stressor effects, HSP90 and HSP70 are considerably upregulated to facilitate the early repair of the damaged proteins (Basu et al. 2002). HSP pose maturation signals and peptides to antigen presenting cells by receptor-mediated interactions. The potential signals released by stressed, infected, damaged, necrotic and neoplastic tissues are also stimulated by endogenous HSP or known as heat shock cognate proteins (HSC). After signals were released from necrotic cells, an inflammatory response occurs by initiating a cascade of components of the acute inflammatory response such as HSP. These HSP stimulate inflammatory mechanism by delivering intra-cellular peptides released from damaged cells, as complexes to antigen presenting cells for activation of T-lymphocytes. Dendritic cells and natural killer T-cells are activated by HSP to increase the presentation of antigens to effector cells, so that both T-cell and humoral responses against their specific antigens are augmented (Segal et al. 2006). Besides forming complexes with non-self proteins such as toxin antigens from the necrotic cells, these HSP also play roles in mediating cytokine production and activating macrophages (Breloer et al. 1999).

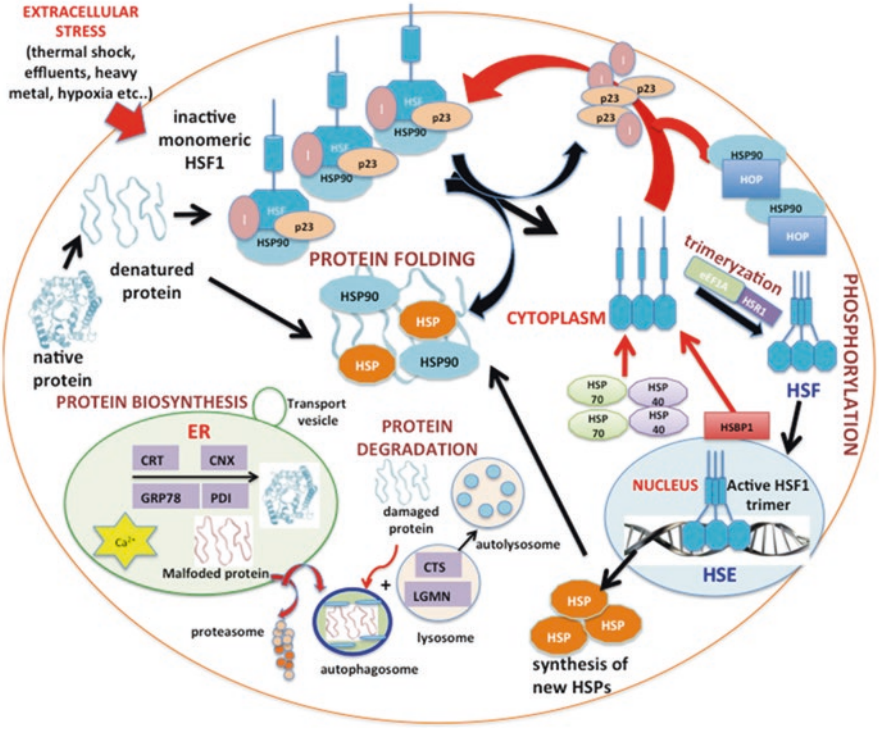

Fig. 9.1 Schematic representation of the mechanism of HSF1 (Heat Shock Factor 1) in HSP (Heat Shock Proteins) induction under environmental stress conditions (adapted from Rupik et al. 2011 and Liu et al. 2013)

In normal physiological condition (Fig. 9.1), HSF1 is present in the cytoplasm as an inactive monomer in the complex with HSP90, p23 and immunophilin. When exposed to stressors, HSF1 localizes in the nucleus, trimerizes and bind the cis-acting HSE found in the heat shock genes (Fernandes et al. 1994). HSF1 is trimerized by both translation elongation factor (eEF1a) and non-coding RNA, RNA-1 (HSR1) (Shamovsky et al. 2006). Following inducible phosphorylation of serine residues, HSF1 trans-activates HSP genes transcription. HSF1 is released from DNA facilitated by HSBP1 (Satyal et al. 1998) and is converted to a monomeric state with assistance from HSP70, HSP90 and HOP (HSC70/HSP90-organizing protein) (Voellmy 2004). HSP70 and cognate HSC70 work in pairs with co-chaperone protein from the HSP40 family. Newly synthesized HSP are recruited to chaperone the denaturing protein pool and proceed with separate pathways, which include protein biosynthesis and protein folding for cellular restoration and cytoprotection. HSP also promote proteolytic degradation of critically damaged proteins by transporting them to targeted organelles (lysosome, autophagosome, autolysosome).

9.1.4 Application of HSP Based Health Therapy for Aquatic Organisms

One of the major obstacles to increase aquaculture production is the outbreak of infectious diseases. Therefore, disease control becomes the main target of aquaculture health management. Among different methods of disease control, the use of vaccine is considered as invaluable tool to protect aquaculture species. Recently, HSP-based vaccines have been developed to restrict pathogenic infections in aquatic organism. HSP derived from pathogen have been used in vaccine production and their efficiency has been proved against bacteria and parasites (Wilhelm et al. 2006; Dang et al. 2011; Ho et al. 2014; Josepriya et al. 2015). The use of recombinant HSP60 and HSP70 derived from pathogenic bacterium, *Piscirickettsia salmonis*, as antigens have enhanced cell protection in infected salmonids (Wilhelm et al. 2006). In another study, a DNA vector carrying HSP70 of parasite *Cryptocaryon irritans* which was used as vaccine showed effective protection against this parasite in spotted grouper (Josepriya et al. 2015). However, HSP vaccination may not always protect aquaculture species from pathogens. Recombinant and DNA vaccines based on HSP60 and HSP70 derived from the bacterium *Flavobacterium psychrophilum* showed no significant protection in rainbow trout *Oncorhychus mykiss* (Plant et al. 2009). In fact, the expression of different antigens following life cycles of bacteria and parasites is the biggest challenge for the development of HSP-based vaccines (Hølvold et al. 2014).

In addition to vaccination approaches, exogenous HSP stimulation has been reported as potential application of HSP in protecting aquaculture species. Normally, species is exposed to some form of stressors to induce HSP (Roberts et al. 2010). Non-lethal heat shock is widely used as stimulator to increase the production of HSP. Non-lethal heat shock has been reported to promote expression of HSP and protect various host against pathogens and thermal stress such as *Artemia franciscana* (Clegg et al. 2000; Sung et al. 2007; Norouzitallab et al. 2015), *Penaeus monodon* (De la Vega et al. 2006), *Cyprinus carpio* (Sung et al. 2014), *Perna viridis* (Aleng et al. 2015) and *Penaeus vannamei* (Junprung et al. 2017).

Apart from temperature, HSP are induced in aquaculture species treated with different compounds. The product Tex-OE®, a compound extracted from prickly pear fruit, *Opuntia ficus indica*, has been indicated to trigger HSP70 in a variety of animals (Baruah et al. 2014). In aquaculture species, Tex-OE® was shown to enhance production of HSP70 that protect angel fish *Pterophyllum scalare*, common carp *Cyprinus carpio* and brine shrimp Artemia *franciscana* from abiotic and biotic stressors (Camilleri 2002; Sung et al. 2012; Baruah et al. 2014). Recently, a soluble variant of Tex-OE®, known as Pro-Tex® has been commercially produced for aquaculture usage (Baruah et al. 2012). The main target of Pro-Tex® in aquaculture is to reduce stresses in salmon and sea bass under transport (Roberts et al. 2010). Also, Pro-Tex® is proven to stimulate basal HSP70 synthesis and stress reduction in brine shrimp *Artemia franciscana*, common carp *Cyprinus carpio* and yellowtail kingfish *Seriola lalandi* (Baruah et al. 2012; Boerrigter et al. 2014).

Another compound, carvacrol is also reported to be a potential HSP inducer. The carvacrol is a phenolic compound and an approved food component that is present in essential oils derived from herbs such as oregano and thyme (Skoula et al. 1999). Carvacrol treatment indicated improvement in the production of HSP72 (belonging to HSP70 family) that increases resistance of artemia larvae against thermal stress or pathogenic *Vibrio harveyi* (Baruah et al. 2017).

9.2 Conclusions

Although the role of HSP/chaperone system has been widely studied, their information in aquatic organisms is hardly accessible. A rapid progress in medical and veterinary research has accelerated the process in obtaining fundamental knowledge of HSP genes, their regulation and the effects of their products for normal cellular homeostasis and cell signaling and immunity defense for cytoprotection. Besides the fundamental role of host HSP in facilitating host immune response, HSP in pathogens are also the major antigens of the pathogens themselves (Zugel and Kaufman 1999). This has led to the development of utilizing pathogen HSP as vaccines in the host system to specifically fight against these pathogens. The recent application of this pathogen-derived HSP vaccines have proven their potential value in the stress and health management of aquatic organisms, particularly aquacultured animals. In addition to HSP vaccines, HSP-induced compounds as Tex-OE® and Pro-Tex® were widely used in aquaculture to enhance HSP expression and protect aquaculture species from stresses. Previously, stressor effects in animals have been evaluated based on conventional and lethal method such as histology, haematology, and clinical chemistry, which are lacking with regards of their sensitivity, duration and cost of the tests. The roles of HSP are not only limited to immune response mediation, but also vital as an environmental monitoring tool for pollutant level assessment via the expression level of HSP (Iwama et al. 2004). Moreover, the valuable properties of HSP such as their inducible nature against a wide range of stressors and their main function as cellular protective machinery, together with the rapid advance in genomic and proteomic technologies have revolutionized risk assessment methods by using HSP as first tier biomarker. Although the expression of HSP genes or proteins has proved promising to understand the detrimental effects of stressors, studies associated with stress genes activation to immunity mechanism pathways in aquatic organism is lacking. Further, the convergence of multiple cellular signalling pathways further complicates the prediction of their main mode of action against stressors. This chapter suggested that the mechanism and pathways of HSP in response to stressor are neither exclusive to stressors nor species, and is indisputably more complex than envisaged. Future studies should be directed towards understanding the mechanism of HSP in a larger number of aquatic organisms towards various extracellular stressors. Research focus could also be directed towards the integration of HSP expression profiling with multidisciplinary studies examining various stages of the whole organisms ranging from molecular, cellular, tissue to physiological levels.

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Chapter 10
Heat Shock Proteins in Aquaculture Disease Immunology and Stress Response of Crustaceans

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Abstract Heat shock proteins (HSP) are ubiquitously expressed proteins for cell growth and viability in all living organisms. The expression and regulation system of HSP is the basis of self-defense and stress response for organisms to response to various internal and external environmental stresses. In this review, recent investigations in HSP of crustaceans are described, which examine roles of HSP in protection of crustaceans from various stress influences. The review also summarizes current understanding of HSP functions in crustaceans' defense response to pathogens infections and other environmental and physiologic stresses. HSP have wilder roles in health of crustaceans, in relation to the immune response to various stressors.

Keywords Aquaculture disease · Crustaceans · Environmental stress · Heat shock proteins · Immunity

Abbreviations

AHPND	Acute hepatopancreatic necrosis disease
ATP	Adenosine triphosphate
CMNV	Covert mortality nodavirus
DNA	Deoxyribonucleic acid
EDCs	Endocrine disruptor chemicals

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H_2O_2,	Hydrogen peroxide
HSC70	Heat shock cognate 70
HSP	Heat shock proteins
HSP70	Heat shock protein 70
IMNV	Infectious myonecrosis virus
kDa	Kilodaltons
NBD	Nucleotide-terminal ATP binding domain
NLHS	Non-lethal heat shock
NP	4-nonylphenol
OP	4–40-octylphenol
PCP	Pentachlorophenol
ProPO	Prophenoloxidase
SBD	Substrate binding domain
SHSP	Small heat shock proteins
UV	Ultraviolet
WSSV	White spot syndrome virus
YHV	Yellow head virus
γ-HCH	γ-hexachlorocyclohexane

10.1 Introduction

Heat shock proteins (HSP), a family of ubiquitously expressed proteins, are known as major actors of the heat shock response (Ritossa 1962; Tissiéres et al. 1974), and are conserved in numerous different kinds of organisms. Many HSP types have been described and constitute a superfamily of proteins ranging from 10 to 110 kilodaltons (kDa). And HSP are categorized into six major families according to their molecular weights, homologies, and functionalities, namely HSP100, HSP90, HSP70, HSP60 or GroEL/ES, HSP40 or J-proteins, and HSP20 or sHSP (small heat shock proteins) (Ratheesh et al. 2012; Feng et al. 2013; Ahmad et al. 2015). As HSP play an important role in protecting organisms from almost any sudden change in the cellular environment that induces protein damage (Welch 1992; Hendrick and Hartl 1993; Sanders 1993; Craig et al. 1994). Various stressors (Table 10.1), such as changes in ambient temperature, organic pollutants, heavy metals stress, toxicants exposure, oxygen deficiency, osmotic stress, ultraviolet radiation, and pathogenic infections, etc., induce the synthesis of HSP. Induction of HSP synthesis and increase in HSP content (up to 15% of the total cellular protein content) by a range of cellular insults (Welch 1993) mainly act as intracellular molecular chaperones, and protecting protein structure and folding under stress condition (Chebotareva et al. 2017). And they are also able to assist with numerous reparative processes including the refolding of denatured proteins, removal of irreparably damaged proteins, translocating proteins into membrane-bound cell compartments and contributing to disease resistance (Table 10.2) (Kampinga and Craig 2010; Junprung et al.

Table 10.1 HSP expression in various aquaculture diseases and stress response in crustaceans

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
HSP100	*Tigriopus japonicus*	Heat exposure	Marginal upregulation at different temperature ranges	Rhee et al. (2009)
HSP90	*Homarus americanus*	Equivalent temperature shifts	Significant inducted expression in *hsp90*	Spees et al. (2002)
	Homarus americanus Nephrops norvegicus	Acute thermal stress, osmotic stress, molting stress	Significant induced higher expression in *hsp90*	Chang (2005)
	Metapenaeus ensis	Exogenous estradiol-17β	Expression of Hsp90 induced by estradiol-17β	Wu and Chu (2008)
	Fenneropenaeus chinensis	Heat shock and hypoxia	*HSP90* mRNA levels were sensitively induced by heat shock and hypoxia	Li et al. (2009a)
	Tigriopus japonicus	Heat exposure	Marginal upregulation at different temperature ranges	Rhee et al. (2009)
	Portunus trituberculatus	Heat or cold shock, hypoosmotic and hyperosmotic stresses, Cu^{2+} stress	Temperature and Cu^{2+} induced significantly up- or down-regulated expression of *HSP90s* in different tissues; low and high salinity induced lower expression levels of *HSP90s*;	Zhang et al. (2009)
	Penaeus monodon	Heat treatment	Significant induced higher expression in *hsp90*	Jiang et al. (2009)
	Penaeus monodon	Oxidative stress: endosulfan and deltamethrin	Endosulfan exposure has a significant time effect on *hsp90* level; deltamethrin did not show any significant effect on HSP expression	Dorts et al. (2009)
	Penaeus monodon	Heat shock, *Vibrio harveyi* injection	Significant induced expression in *hsp90*	Rungrassamee et al. (2010)

(continued)

Table 10.1 (continued)

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
	Daphnia magna	Environmental stresses (cyanobacteria, predation from fish, toxic compounds, temperature)	Significant increase in the level of HSP60s in response to raised temperature in females	Mikulski et al. (2011)
	Calanus finmarchicus	Diapause, handling (deep and shallow, ice-chilled) stress	Significant induced expression in *Hsp90*	Aruda et al. (2011)
	Exopalaemon carinicauda	pH and ammonia-N stresses	*HSP90* could be induced by various stresses from environment	Li et al. (2012)
	Eriocheir sinensis	Salinity stress	Both low and high salinities markedly stimulated expression of *Hsp90*	Sun et al. (2012)
	Litopenaeus vannamei	Acute thermal stress, pH challenge, and heavy metal exposure	*S*ignificantly induced by thermal expression in *Hsp90*	Qian et al. (2012)
	Portunus trituberculatus	Salinity stress	Significant induced expression in *hsp90*	Bao et al. (2014)
	Eurytemora affinis	Reproductive condition stress	*Hsp90A* transcript levels gradually increased during oogenesis in ovigerous females	Boulangé-Lecomte et al. (2014)
	Macrobrachium rosenbergii	Various pathogenic infections	Expression level was up-regulated	Chaurasia et al. (2016)
	Penaeus vannamei	Acute and chronic non-lethal heat shock, and acute hepatopancreatic necrosis disease causing strain of *Vibrio parahaemolyticus* (VP_{AHPND})	*HSP90* was induced by non-lethal heat shock, and chronic non-lethal heat shock enhance shrimps tolerance to VP_{AHPND} infection	Junprung et al. (2017)
HSP70	*Oniscus asellus*	Organic chemicals (B[a]P, PCB52, g-HCH, PCP)	Significant and inducted *hsp70* response after about 24 h of exposure to organic chemicals	Köhler et al. (1999)

(continued)

Table 10.1 (continued)

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
	Macrobrachium malcolmsonii	Hg and Cu	Transient and differential expression of *hsp70* between gill and hepatopancreas	Yamuna et al. (2000)
	Homarus americanus	Equivalent temperature shifts	Significant inducted expression in *hsp70*	Spees et al. (2002)
	Macrobrachium rosenbergii	Heat shock stress	*hsp70* expression was induced by 35 °C heat shock	Liu et al. (2004)
	Oniscus asellus	Metals	Higher *hsp70* levels	Arts et al. (2004)
	Porcellio scaber	Metals	Lower *hsp70* levels	
	Homarus americanus Nephrops norvegicus	Acute thermal stress, osmotic stress, molting stress	Significant induced heat-shock, hypo- and hyper-osmotic response	Chang (2005)
	amphipods	Cadmium chloride and temperature stresses	Induced by both temperature and toxic stresses	Timofeev et al. (2008)
	Chasmagnathus granulatus	heat-shock and water-deprivation stresses	Increasing expression in Hsp70	Frenkel et al. (2008)
	Artemia franciscana	Abiotic stress and *Vibrio* challenge	*Hsp70* accumulation induced by abiotic stress and enhanced resistance to infection by *Vibrio campbellii*	Sung et al. (2008)
	Tigriopus japonicus	Environmental toxicants (heat, heavy metals and endocrine-disrupting chemicals (EDCs))	Significant upregulation of *Hsp70* mRNA expression; Some EDCs caused significant down regulation expression in *Hsp70*	Rhee et al. (2009)
	Gammarus pulex	Dissolved humic substances (HSs)	Significant increased expression in HSP70	Bedulina et al. (2010)
	Artemia franciscana	*Vibrio* challenge	Significant induced expression in HSP70	Baruah et al. (2010)
	Penaeus monodon	Heat shock, *Vibrio harveyi* injection	Significant induced expression in *hsp70*	Rungrassamee et al. (2010)
	Litopenaeus vannamei	Bacterial challenge	*HSP70* was induced in different tissues after different bacteria injection	Zhou et al. (2010)

(continued)

Table 10.1 (continued)

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
	Niphargus	Thermal stress	Significant induced expression in *Hsp70*	Colson-Proch et al. (2010)
	Fenneropenaeus chinensis	Heat shock and heavy metal treatments	Heat shock and copper induced up-regulation of *Hsp70* and *Hsc70*; cadmium treatment did not induce the expression of *Hsp70*, but caused down-regulation of *Hsc70*	Luan et al. (2010)
	Portunus trituberculatus	Bacterial challenge	The expression of *Hsp70* induced by *Vibrio alginolyticus*	Cui et al. (2010)
	Daphnia magna	Environmental stresses (cyanobacteria, predation from fish, toxic compounds, temperature)	The different types of environmental stress induced significant stress response in females; significant interaction between the effect of thermal shock and the effect of sex on the level of HSP70	Mikulski et al. (2011)
	Calanus finmarchicus	Diapause, handling (deep and shallow, ice-chilled) stress	Significant induced expression in *Hsp70s*	Aruda et al. (2011)
	Procambarus clarkii	Extreme light cycles	The expression of *HSP70* showed up-regulation	Velázque-Amado et al. (2012)
	Eriocheir sinensis	Salinity stress	Both low and high salinities markedly stimulated expression of *Hsp70*	Sun et al. (2012)
	Chasmagnathus granulatus	Food odor stimuli, visual danger stimulus	Significant raised expression in HSC/HSP70	Frenkel et al. (2012)
	Litopenaeus vannamei	Acute thermal stress, pH challenge, and heavy metal exposure	*Hsp70* was significantly induced by thermal stress, pH stress, iron and zinc stimulation	Qian et al. (2012)
	Pachygrapsus marmoratus	Thermal stress	Hsp70 production altered by increasing temperature	Madeira et al. (2012)

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Table 10.1 (continued)

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
	Scylla paramamosain	Bacterial, osmotic, and thermal stress	*Hsp70* was inducible by bacterial, osmotic, and thermal stress	Yang et al. (2013)
	Litopenaeus vannamei	Nitrite-N stress	HSP70 expression level significantly up-regulated to protect the hemocyte against nitrite stress	Guo et al. (2013)
	Scylla serrata	Temperature, pathogen, salinity, and nitrite stress	The expression level of *Hsp70* significantly increased under different stresses	Fu et al. (2013)
	Litopenaeus vannamei	Abrupt heat shock, and *Vibrio* infection	The synthesis of shrimp Hsp70 mRNA and protein were induced by heat shock	Loc et al. (2013)
	Procambarus clarkii	Acoustic stimuli	significant increase in expression of Hsp70 protein	Celi et al. (2013)
	Gammarus pulex	Multiple microsporidian infections and temperature stress	Significant effects of temperature and microsporidian infection on *hsp70*	Grabner et al. (2014)
	Tigriopus japonicus	Heavy metals stress	The expression of *hsp70* was substantially modulated by exposure to heavy metals	Kim et al. (2014)
		Thermal stress, and bacterial challenge	The expressions of Hsc70s showed substantial obvious heat- and bacterial-inducible regulation	Xiu et al. (2014)
	Portunus trituberculatus	Salinity stress	Significant induced expression in *hsp70*	Bao et al. (2014)
	Pachygrapsus marmoratus	Synergistic effects of temperature, salinity and pH	Heat stress response is altered by the synergistic effect of variables	Madeira et al. (2014)

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Table 10.1 (continued)

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
	Gammarus pulex	Thermal stress	Heat inducible expression in *hsp70* and *hsc70* in early life stages and highly expressed in adults; significant inducible expression of *hsp70* in species along the latitudinal thermal gradient	Cottin et al. (2015)
	Antarctic krills (*Euphausia superba* and *E. crystallorophias*)	Thermal shocks	Hsp70s and Hsc70s of two krill species have different responses to temperature	Cascella et al. (2015)
	Paracyclopina nana	UV radiation	Significantly increased in response to UV radiation	Won et al. (2015)
	Artemia fransciscana	Non-lethal heat shock and *Vibrio* challenges	HSP70 levels are elevated sequentially in *Artemia* under on-lethal heat shock, and increased protection of *Artemia* against *Vibrio* challenges	Norouzitallab et al. (2015)
	Palaemon elegans *Palaemon serratus*	Thermal stress	No significant different between the congeners, no significant differences in HSP70 levels along the temperature trial	Madeira et al. (2015)
	Artemia sinica	CO_2-driven seawater acidification	Up-regulated in all treatments	Chang et al. (2016)
	Daphnia magna	Cadmium and heat stress	Cadmium and heat stress caused induction of Hsp70 in individuals	Haap et al. (2016)
	Eriocheir sinensis	*Vibrio* challenges	*Hsc70s* were significantly induced by *Vibrio* challenges	Li et al. (2016)
	Macrobrachium rosenbergii	Various pathogenic infections	Expression level was up-regulated	Chaurasia et al. (2016)

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Table 10.1 (continued)

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
	Artemia franciscana	Cd and Zn acute exposures, and non-lethal heat shock	Significantly altered HSP levels; Cd exposure induced HSP production, while Zn exposure had no significant effects on HSP production	Pestana et al. (2016)
	Gammarus pulex	Temperature and ammonia treatments	Significant increase in *hsp70* mRNA expression by temperature treatments, NH3 concentration had no effect on *hsp70* mRNA synthesis across temperatures	Henry et al. (2017)
	Penaeus vannamei	Acute and chronic non-lethal heat shock, and acute hepatopancreatic necrosis disease causing strain of *Vibrio parahaemolyticus* (VP_{AHPND})	*HSP70* was induced by non-lethal heat shock, and chronic non-lethal heat shock enhance shrimps tolerance to VP_{AHPND} infection	Junprung et al. (2017)
	Amphipods *Eulimnogammarus verrucosus and E. cyaneus*	Acute thermal stress	Hsp70 was induced by acute thermal stress, and the significantly higher level of Hsp70 in the thermotolerant *E. cyaneus*, compared with *E. verrucosus*	Bedulina et al. (2017)
HSP60	*Tigriopus japonicus*	Heat exposure	Marginal upregulation at different temperature ranges	Rhee et al. (2009)
	Litopenaeus vannamei	Bacterial challenge	*HSP60* was significantly up-regulated in different tissues after bacterial challenge	Zhou et al. (2010)
	Litopenaeus vannamei	Environmental stress and pathogenic infection	The expression of *HSP60* was variable under different stresses	Huang et al. (2011)

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Table 10.1 (continued)

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
	Daphnia magna	Environmental stresses (cyanobacteria, predation from fish, toxic compounds, temperature)	The different types of environmental stress induced significant stress response in females; significant interaction between the effect of thermal shock and the effect of sex on the level of HSP60	Mikulski et al. (2011)
	Litopenaeus vannamei	Acute thermal stress, pH challenge, and heavy metal exposure	*Hsp60* was significantly induced by heat strss, pH stress, cadmium and manganese exposure	Qian et al. (2012)
	Portunus trituberculatus	Salinity stress	Salinity challenges significantly altered the expression of HSP60 at mRNA and protein level	Xu and Qin (2012)
	Portunus trituberculatus	Salinity stress	Significant induced expression in *hsp60*	Bao et al. (2014)
	Paracyclopina nana	UV radiation	*Hsp60* was significantly increased in response to UV radiation	Won et al. (2015)
	Penaeus monodon	pH challenge, osmotic stress, and heavy metal exposure	Significant induced expression in *hsp60* and *hsp10*	Shi et al. (2016)
	Macrobrachium rosenbergii	Various pathogenic infections	Expression level was up-regulated	Chaurasia et al. (2016)
HSP40	*Tigriopus japonicus*	Heat exposure	Marginal upregulation at different temperature ranges	Rhee et al. (2009)
	Paracyclopina nana	UV radiation	Significantly increased in response to UV radiation	Won et al. (2015)
sHSP	*Artemia franciscana*	Long-term anoxia	Substantial amounts of p26 translocated into nuclei of anoxic brine shrimp embryos	Clegg et al. (2000)
	Tigriopus japonicus	Endocrine disruptors	*Hsp20* gene expression varied amongst endocrine-disrupting chemicals	Seo et al. (2006)

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Table 10.1 (continued)

HSP	Species	Type of aquaculture diseases or stress factors	Stress response of HSP	References
	amphipods	Cadmium chloride and temperature stresses	Induced by both temperature and toxic stresses	Timofeev et al. (2008)
	Penaeus monodon	White spot syndrome virus (WSSV) infection	*HSP21* gene showed down-regulation after WSSV infection	Huang et al. (2008)
	Tigriopus japonicus	Heat exposure	Marginal upregulation at different temperature ranges	Rhee et al. (2009)
	Gammarus pulex	Dissolved humic substances (HSs)	Significant increased expression in sHsp	Bedulina et al. (2010)
	Penaeus monodon	Heat shock, *Vibrio harveyi* injection	Significant induced expression in *hsp21*	Rungrassamee et al. (2010)
	Calanus finmarchicus	Diapause, handling (deep and shallow, ice-chilled) stress	Significant induced expression in *sHsp*	Aruda et al. (2011)
	Macrobrachium rosenbergii	Necrosis virus infection	The expression of *HSP37* is strongly up-regulated after necrosis virus challenge	Arockiaraj et al. (2012)
	Penaeus monodon	Salinity stress	Significant increase in hsp21 expression levels in muscle and gut tissues	Shekhar et al., (2013)
	Tigriopus japonicus	Heavy metals stress	The expression of *hsp20* was substantially modulated by exposure to heavy metals	Kim et al. (2014)
	Paracyclopina nana	UV radiation	Significantly increased in response to UV radiation	Won et al. (2015)

2017). Currently, numerous members of the HSP families have been thoroughly studied and analyzed in various organisms, and protein-protein interaction network among HSP has been graphed (Fig. 10.1). HSP are almost ubiquitously expressing in normal physiological processes of all living organisms, but the expression levels of HSP are induced up- or down-regulation when organisms or cells are exposed to various physiological and environmental stress conditions. In addition, the different members of HSP families have different expression patterns, and some members from different family participate in interaction effects. Experimental studies have proved that overexpression and/or inhibition of HSP play an important role in

Table 10.2 The pleiotropic effects of HSP in crustaceans

Species	HSP	Effects	References
Tigriopus japonicus	HSP105/ HSP90/ HSP70	Involved in thermotolerance and protective response to xenobiotics	Rhee et al. (2009), Kim et al. (2014)
	HSP20	Stress response to endocrine disruptors	Seo et al. (2006)
Homarus americanus	HSP90/ HSP70/ HSC70	Response to equivalent thermal shifts Participate in in multiple signal transduction pathways Involved in the regulation of ecdysteroid receptors in claw muscle cells responsive to molting hormones	Spees et al. (2002). Spees et al. (2003), Chang (2005)
Oniscus asellus	HSP70	Protective response to organic chemicals, metals	Köhler et al. (1999), Arts et al. (2004)
Procambarus clarkii	HSP70	Help medial giant axon maintain essential structures and functions	Sheller et al. (1998)
		Regulate the folding of core clock proteins	Velázque-Amado et al. (2012)
Macrobrachium malcolmsonii	HSP70	Protective response to metals	Yamuna et al. (2000)
Artemia franciscana	sHSP	Protection and repair of nuclear components	Clegg et al. (2000)
	HSP70	Enhanced thermotolerance and resistance to pathogenic infection Participate in protective immunity against pathogenic vibrios	Sung et al. (2008), Baruah et al. (2010), Baruah et al. (2013), Baruah et al. (2014), Norouzitallab et al. (2015)
Macrobrachium rosenbergii	HSP70/ HSC70	Protect against muscle damage, play role in growth and development	Liu et al. (2004)
	HSP37	Molecular chaperone, involved in the immune responses against IHHNV challenge	Arockiaraj et al. (2012)
Metapenaeus ensis	HSP90	Involved in the regulation of vitellogenin synthesis	Wu and Chu (2008)
Porcellio scaber	HSP70	Stress response to metals pullution	Arts et al. (2004)
Gammarus lacustris *Eulimnogammarus cyaneus* *Eulimnogammarus verrucosus*	HSP70/ sHSP	Involved in stress-defense system	Timofeev et al. (2008)
Chasmagnathus granulatus	HSP70/ HSC70	Involved in sensory processing or activation of olfactory receptor neurons	Frenkel et al. (2008), Frenkel et al. (2012)

(continued)

Table 10.2 (continued)

Species	HSP	Effects	References
Penaeus monodon	HSP21	Participate in protection of cells' apoptosis	Huang et al. (2008), Rungrassamee et al. (2010)
	HSP90	Play roles in ovarian maturation and defending the circumstantial temperature elevation; Participate in preventing protein denaturation and/or aggregation	Jiang et al. (2009), Dorts et al. (2009), Rungrassamee et al. (2010)
	HSP70	Immunity response against pesticide or pathogen	Dorts et al. (2009), Rungrassamee et al. (2010)
	HSP10/ HSP60	Molecular chaperone complex, participate in environmental stress responses	Shi et al. (2016)
Fenneropenaeus chinensis	HSP90 HSP70/ HSC70	Play roles for shrimp to cope with environmental stress Protecting cells from damage	Li et al. (2009a), Luan et al. (2010)
Portunus trituberculatus	HSP90s HSP70	Participate in transcriptional regulation and stress response Involved in immune reactions	Zhang et al. (2009), Cui et al. (2010)
	HSP60	Mediating the salinity stress	Xu and Qin (2012), Bao et al. (2014)
Gammarus pulex	sHSP/ HSP70 HSC70/ HSP70	Act as a primordial exogenous trigger for the development of anti-stress systems in exposed organisms; Protective effect for the host against acute temperature stress	Bedulina et al. (2010), Grabner et al. (2014), Cottin et al. (2015), Henry et al. (2017)
Litopenaeus vannamei	HSP60/ HSP70 HSC70/ HSP90	Mediating the immune responses to environmental stress and pathogenic infection, protect the hemocyte against nitrite stress; Adaptive response to thermal stress	Zhou et al. (2010), Huang et al. (2011), Qian et al. (2012), Guo et al. (2013), Loc et al. (2013)
Niphargus	HSP70	Involved in dealing with this short-term and small-scale stress	Colson-Proch et al. (2010)
Daphnia magna	HSP60/ HSP70/ HSP90	Involved in stress-defense system	Mikulski et al. (2011), Haap et al. (2016)
Calanus finmarchicus	HSP21/ HSP22/ HSP90/ P26/ HSP70s	Play roles in short-term stress responses, stress tolerance and in protecting proteins from degradation	Aruda et al. (2011)
Cherax quadricarinatus	HSP70	Regulating spermatogenesis	Fang et al. (2012)

(continued)

Table 10.2 (continued)

Species	HSP	Effects	References
Exopalaemon carinicauda	HSP90	Involved in environmental adaptation	Li et al. (2012)
Eriocheir sinensis	HSP90/ HSP70	Play role in salinity tolerance	Sun et al. (2012)
	HSC70s	Involved in development al regulation	Li et al. (2016)
Artemia	HSP21/ HSP22	Prevents spontaneous termination of diapause and provides stress protection to encysted embryos	King and MacRae (2012)
	HSP70	Increases tolerance to metal exposure	Pestana et al. (2016)
Pachygrapsus marmoratus	HSP70	Participate in hormonal regulation and thermal tolerance adaptation	Madeira et al. (2012), Madeira et al. (2014)
Scylla paramamosain	HSP70	Play role in innate immune responses	Yang et al. (2013)
Scylla serrata	HSP70	Disease resistance factor	Fu et al. (2013)
Macrobrachium nipponense	HSC70s	Mediating responses to heat-shock and bacterial challenge	Xiu et al. (2014)
Eurytemora affinis	Grp78/ HSP90A	Responsible for differential stress tolerance	Boulangé-Lecomte et al. (2014)
Euphausia superba and *Euphausia crystallorophias*	HSP70	Adaptaion to cold stress	Cascella et al. (2015)
Paracyclopina nana	HSP10/ HSP40/ HSP60/ HSP70	Defense and recover cellular damage	Won et al. (2015)
Palaemon elegans and *Palaemon serratus*	HSP70/ HSC70	HSP counteracts the effects of thermal challenge	Madeira et al. (2015)
Artemia sinica	HSP70	Participate in cellular stress responses	Chang et al. (2016)
Macrobrachium rosenbergii	HSP60/ HSP70/ HSP90	Protecting cells against pathogens	Chaurasia et al. (2016)
Penaeus vannamei	HSP70/ HSP90	Play crucial roles in bacterial defense	Junprung et al. (2017)
Eulimnogammarus verrucosus and *Eulimnogammarus cyaneus*	HSP70	Mediating responses to heat-shock	Bedulina et al. (2017)

HSP heat shock protein; *sHSP* small heat shock proteins

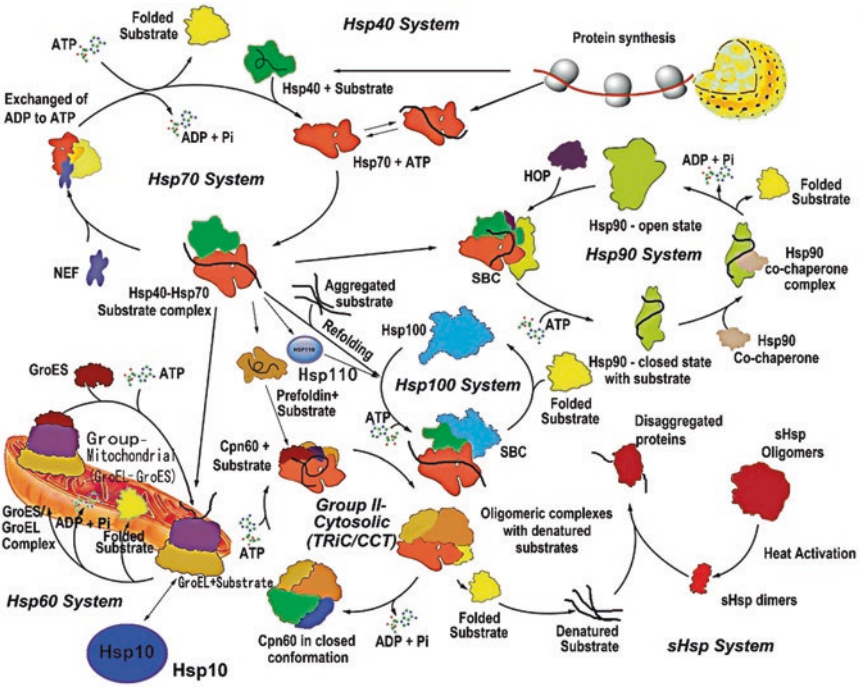

Fig. 10.1 A functional HSP (HSP100, HSP90, HSP70, HSP60, HSP40, and small HSP family) molecular network (Cited from http://pdslab.biochem.iisc.ernet.in/hspir/index.php; Mogk et al. 2015; Bar-Lavan et al. 2016)

maintaining the tolerance and cell viability under above-described stress conditions (Pathan et al. 2010). In recent years, researchers have investigated that the heat shock chaperonin are involved in autoimmune and innate immune response in several species including crustaceans (Tsan and Gao 2004; Cerenius et al. 2010; Chaurasia et al. 2016; Junprung et al. 2017). The activity of HSP is one of the most effective organism protection mechanisms.

10.1.1 The HSP100 Family

The HSP100 family, a class of molecular chaperones belongs to the AAA+ superfamily of ATPases (Bukau et al. 2006), have the ability to solubilize almost any protein that becomes aggregated after severe stress. HSP100 proteins are not required under normal conditions and induced by extreme physiological or environmental stresses. They are categorized into two classes: class I proteins with two AAA+ modules and class II chaperones. HSP100 types A–D, HSP104, bacterial ClpB and their distant relatives ClpA, ClpC belongs to class I whereas HSP100 types M, N, X, Y, ClpX and HslU are a part of class II chaperones (Agarwal et al.

2001; Doyle and Wickner 2009). Hsp100 proteins are composed of 5 specific domains: (i) amino (N)–terminal domain, (ii) nucleotide-binding domain (NBD) 1, (iii) middle domain, (iv) NBD2, and (v) carboxyl (C)–terminal domain (Agarwal et al. 2001). HSP100 chaperones differ in the number of AAA domains (one or two) per protomer and the presence of extra domains, which provide functional specificity by controlling substrate interactions (Mogk et al. 2015).

Members of the HSP100 family are constitutively expressed proteins, but they are also induced expression by environmental stress stimulation. Stressors inducibility of *hsp100* gene and HSP100 protein have been noted in various organisms examined thus far. For instance, the expression levels of HSP105 in the intertidal copepod *Tigriopus japonicus* is upregulation at different temperature ranges (Rhee et al. 2009), which indicating that this gene participate in stress response of thermotolerance. Another member of the HSP100 family, HSP110, possesses HSP70-indepensent functions and preventing protein aggregation (Bar-Lavan et al. 2016). HSP110 can also form heterodimers with HSP70 and is suggested to function as its co-chaperone in protein disaggregation (Bracher and Verghese 2015; Nillegoda and Bukau 2015).

10.1.2 The HSP90 Family

The HSP90 proteins, named according to the 90 kDa average molecular mass of their members, are highly conserved molecular chaperones that contributing to the folding, maintenance of structural integrity and proper regulation of a subset of cytosolic proteins (Rutherford and Lindquist, 1998; Picard, 2002; Chen et al., 2006). HSP90 is one of the most studied and abundant proteins of eukaryotic cells, comprising 1–2% of total proteins under non-stress conditions. HSP90 has a tendency to form complexes with a variety of other proteins and it alone may be able to interact with more than 400 different proteins, which make it as a multifunctional protein (Csermely and Kahn, 1991; Buchner, 1999; Young et al., 2001; Zhao et al., 2005). While ubiquitously expressed in unstressed normal cells, the HSP90 complex assists in the folding and function of a variety of proteins (client proteins). HSP90 stabilizes its 'client' proteins in conformations that would otherwise be prone to misfolding and potentiates their capacity to be activated in the proper time and place, by associations with partner proteins, ligand binding, post-translational modifications and correct localization (Buchner, 1999; Mayer and Bukau, 1999; Young et al., 2001). HSP90 thus forms a hub that controls numerous important signalling pathways in eukaryotic cells (Fig. 10.1), making the HSP90 chaperone system central to cellular regulation and function (Taipale et al. 2014). Currently, extensive studies on HSP90s of crustaceans indicated that there are pleiotropic functions in protecting cell survival and self-defense after stress (Aruda et al. 2011; Qian et al. 2012; Boulangé-Lecomte et al. 2014; Chaurasia et al. 2016; Junprung et al. 2017). HSP90 exerts its function through the refolding of different proteins and thus contributes to various biological activities under non-stress conditions (Bohen et al., 1995).

Structural studies have identified the ATP-binding site in the N-terminal domain of HSP90 (Csermely et al., 2017), and the middle domain of HSP90 is important for the trapping of the ATP molecule (Kahn et al. 1993; Wegele et al. 2003). Therefore, it is clear that HSP90 protein contains three functional domains, the ATP-binding, protein-binding, and dimerizing domain, and each of these domains plays a crucial role in the functioning of the protein.

10.1.3 The HSP70 Family

Among the HSP, the heat shock protein 70 (HSP70) family members, including the heat shock induced member and the constitutively expressed HSC70 (heat shock cognate 70), are most extensively studied and abundant chaperones in the cell for their characterization and induction in response to environmental stressors in a range of species (Kim et al. 2013). The eukaryotic HSP70 family consists of four monophyletic groups: cytosol, endoplasmic reticulum, mitochondria, and chloroplast HSP70s (Kregel 2002; Bausero et al. 2005). HSP70s act as molecular chaperones, and their expression is regulated by environmental and physiological stressors (Chichester et al. 2015; Pestana et al. 2016; Henry et al. 2017; Bedulina et al. 2017) and non-stressful conditions, such as cell growth, development, and pathophysiological conditions (Behnke and Hendershot, 2014; Tiroli-Cepeda et al., 2014). HSP70 family members serve highly diverse functions, including translocation, folding newly synthesized proteins, degradation of unstable and misfolded proteins, as well as the prevention and dissolution of protein complexes, etc. HSP70 actions are regulated by some co-chaperones, namely HSP40 proteins, NEFs (nucleotide exchange factors), and TPR (tetratricopeptide repeat) co-chaperones (Fig. 10.1). HSP70 can co-operate with HSP100 or HSP110 in protein disaggregation (Glover and Lindquist 1998; Mogk et al. 2015; Finka et al. 2015; Nillegoda and Bukau 2015). In summary, HSP70s are a central hub of the chaperone network carrying very diverse physiological functions in proteostasis (Finka et al. 2015).

10.1.4 The HSP60 Family

The Hsp60 family, also known as the chaperonins with distinct ring-shaped, or toroid (double doughnut) quaternary structures (Quintana and Cohen 2005), comprises two distinct classes that can be differentiated on the basis of sequence alignment and the need for a lid-like co-chaperone (Bar-Lavan et al. 2016). HSP60 protein is present in nearly all prokaryotes and in organelles of eukaryotic cells. Over the last several decades, multiple HSP60 functions have been discovered. HSP60 is mainly involved in protein folding and stress protection in mitochondria and chloroplasts of eukaryotes (Cechetto et al. 2000). Moreover, HSP60 chaperonins appear to play central roles in mammalian defences against pathogenic attack

and responses to damage or stress in addition to normal cell functions (Vabulas et al. 2001). In addition, much of the research on organism protection under environmental stress have indicated the involvement of HSP60 in various stress responses. For instance, HSP60 participates in immune and stress responses under different environmental stresses and stimuli in white shrimp *Litopenaeus vannamei* (Huang et al. 2011). HSP60 plays important roles in salinity stress mediation in swimming crab *Portunus trituberculatus* (Xu and Qin 2012). In addition, HSP60 and HSP10 were involved in the responses to pH challenge, osmotic stress, and heavy metal exposure in black tiger shrimps *Penaeus monodon* (Shi et al. 2016).

HSP60 can binds to unstable folding intermediates. In most cases, HSP10 and ATP are required for further folding of the substrate protein and release from HSP60 (Parsell and Lindquist 1993). HSP10, as the co-chaperonin of HSP60, exters its biological functions by combining with HSP60 protein (Cappello et al. 2014; Boettinger et al. 2015). Recent study (Shi et al. 2016) indicated that HSP60/HSP10 may be combined to produce a chaperone complex with effective chaperone and ATPase activities. In fact, the ATP hydrolysis drives conformational changes of cage, creating a protected environment favoring protein folding (Clare and Saibil 2013). In crustaceans, HSP60/HSP10 chaperone complex can defend organisms against cellular stress and demonstrate how the complex participates in environmental stress responses (Shi et al. 2016).

10.1.5 The HSP40 Family

The HSP40 family proteins are ubiquitously expressed in all organisms ranging from bacteria to higher eukaryotes. The family of HSP40 is characterized by the presence of a conserved J-domain that has approximately 70 amino acid residues and is hence termed as the J-protein in eukaryotes and DnaJ in prokaryotes. HSP40 or J-proteins are further divided into four different types, that is, Type-1, Type-2, Type-3 and Type-4 (Feng et al. 2014). Currently, it is consistent with the idea that the HSP40 family has evolved to aid in the versatility and multi-functionality of the HSP70 chaperone system (Fig. 10.1). Studies indicated that HSP40 protein functions in specific pair with HSP70 protein, the J-domain of HSP40 could make direct contacts with the N-terminal ATPase domain of HSP70, to promote protein folding, protein transport and degradation (Wittung-Stafshede et al. 2003; Hennessy et al. 2005). A recent review demonstrated that HSP40 proteins are involved in cellular stress protection, folding of nascent polypeptides, refolding of denatured or aggregated proteins, protein degradation, and protein translocation, etc., (Cyr and Ramos 2015). The expression levels of HSP40 genes in crustaceans are up-regulation in response to heat exposure or UV radiation (Rhee et al. 2009; Won et al. 2015). However, there are still many unknowns about the molecular chaperone mechanism of the HSP40 family proteins.

10.1.6 The Small HSP Family

Small heat shock proteins (sHSP) are conserved across species, which exists in all living organisms ranging from bacteria to archaea, and are important in stress tolerance. sHSP functions are very diverse and participate in various biological processes, such as the response and adaptation to cell stress, thermotolerance, cell differentiation, and cell apoptosis, etc. (de Jong et al. 1993; Haslbeck and Vierling 2015; Carra et al. 2017) and play an important role in cell survival under conditions of stress (Bakthisaran et al. 2015). Unlike any other HSP family proteins, the sHSP chaperones do not require ATP hydrolysis for its function. sHSP proteins in vivo have the ability to bind proteins undergoing denaturation and prevent their misfolding. sHSP form a part of the cellular chaperone network, preventing aggregation of target proteins and maintaining them in a folding-competent state. sHSP together with molecular chaperones from the HSP70 and HSP100 family serve to maintain "proteostasis" in cells (Fig. 10.1, see review Bakthisaran et al. 2015). A number of studies (Rhee et al. 2009; Arockiaraj et al. 2012; Shekhar et al., 2013; Kim et al. 2014; Won et al. 2015) have demonstrated sHSP are up-regulated in crustaceans responding to various environmental stresses, such as heat exposure, necrosis virus infection, salinity stress, heavy metals stress, and UV radiation, etc. A more recent work (Bar-Lavan et al. 2016) have indicated that sHSP appears to provide a large surface area that can bind and sequester aggregation-sensitive folding intermediates and prevent their aggregation during stress conditions.

sHSP are composed of a non-conserved N-terminal domain (NTD) of variable length, a highly conserved α-crystallin domain (ACD) and a non-conserved short C-terminal domain (CTD) (Kriehuber et al. 2010; Bar-Lavan et al. 2016). Studies on structure and function of sHSP have shown that all three domains play an important role in sHSP oligomerization and function (McDonald et al. 2012; Mainz et al. 2015). Many sHSP have been found to form large oligomeric structures. The NTD and CTD were shown to play a role in sHSP assembly into higher oligomeric structures (Bar-Lavan et al. 2016). The ability to prevent the aggregation of proteins and polypeptides is the most important function of many sHSP in response to particular stress conditions that lead to unfolding of cellular proteins (Bakthisaran et al. 2015). Moreover, the ability of sHSP to interact with cytoskeletal elements and protect those under conditions of stress can prevent the cascade of events leading to cell-death. Within the protein homeostasis network of the cell, sHSP can function as a buffer system to bind unfolding proteins upon stress, protecting them from irreversible aggregation (Haslbeck and Vierling 2015). In addition, phosphorylation of sHSP modulates a variety of their functions in cellular processes such as apoptosis, cell cycle and differentiation, etc. sHSP such as HSP27, α-crystallins, HSP20 and HSP22 are known to get phosphorylated, especially under stress conditions (Hayes et al. 2009; Bakthisaran et al. 2015). Many studies have demonstrated that the expression of many sHSP is dramatically elevated by heat and other stresses, increasing the availability of these chaperones when needed during stress (Haslbeck et al. 2015; Haslbeck and Vierling 2015). Recent study indicated that some sHSP

are constitutively expressed, but their levels can be increased upon diverse stress conditions, supporting their implication in the cell and organismal stress response (Carra et al. 2017).

10.1.7 Aquaculture Diseases and Environmental Stresses of Crustaceans

Crustacean aquaculture represents a major industry in numerous countries around the world. With the increasing extension and intensification of aquaculture farms, new various diseases have recently appeared in commercially exploited species. The globe's crustacean aquaculture industry has been serious threatened by disease outbreaks, mainly caused by various viruses, bacteria, and other environmental stressors, which have resulted in enormous economic losses. Invertebrates lack true adaptive immunity and have developed defense systems that respond against physiological or environmental stresses (Vazquez et al. 2009; Labaude et al., 2017b). During the crustacean aquaculture, organisms are constantly exposed to environmental stimuli and natural and/or anthropogenic stressors. A large number of studies have demonstrated a series of physical (e.g. temperature, salinity, and UV radiation) and chemical (e.g. endocrine disruptor chemicals, heavy metals, hydrocarbons, and other toxicants) stressors that can be damaging to cells of crustaceans. Moreover, in natural ecosystems, multiple environmental forces interact, leading to multi-stress situations (Travis 2003; Dehedin et al. 2013). Crustaceans have an innate immune system and it is the first line of defense may react to natural and/or anthropogenic stimuli, pollutants, and toxins (Kozlowsky-Suzuki et al. 2009; Lauritano et al. 2012). Studies have demonstrated that some metabolic enzymes (e.g. cytochrome P450, glutathione S-transferase, superoxide dismutase, etc.), heat shock proteins, and immune-related proteins in crustaceans can participate in to enhance disease tolerance and to facilitate elimination of compounds from the body of organisms (Cerenius et al. 2010; Lauritano et al. 2012; Junprung et al. 2017).

10.1.7.1 Pathogens and Shellfish Diseases

Shellfish diseases are common and frequently reported in various species of commercially exploited crustaceans. Currently, various pathogens, such as *Vibrio* (Rivera et al. 1999), chitinoclastic bacteria (Powell and Rowley 2005), *Aeromonas* (Xu and Xu 2002), *Spiroplasma* (Wang et al. 2004), Rickettsia-like organism (Wang et al. 2001), Chlamydia-like organism (Sparks 1985), Rhodobacteriales-like organism (Eddy et al. 2007), white spot syndrome virus (WSSV) (Lo et al. 1996), yellow head virus (YHV) (Cowley et al. 2012), infectious myonecrosis virus (IMNV) (Lightner et al. 2012), a microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) (Tangprasittipap et al. 2013), and covert mortality nodavirus (CMNV) (Zhang et al. 2014), etc., have been demonstrated causing a series of diseases in

crustaceans (reviewed in Thitamadee et al. 2016). The *Vibrio* species are ubiquitous throughout the world and are found associated with many marine and freshwater crustaceans. *Vibrio* infections typically cause or produce bacteremias and shell disease (reviewed in Wang 2011). For example, *Vibrio parahaemolyticus* infection has caused acute hepatopancreatic necrosis disease (AHPND) and led to severe mortalities in penaeid shrimp aquaculture (FAO 2014; Tran et al. 2013). Moreover, chitinolytic or chitinoclastic bacteria are generally associated with shellfish disease and result in crustacean's unsuccessful molting (Smolowitz et al. 1992) or septicaemic infections by opportunistic pathogenic bacteria (Vogan et al. 2001). Other pathogens such as Rickettsia-like, Chlamydia-like, spiroplasma, and Rhodobacteriales-like organisms' infection in crustaceans have caused serious stress or fatal disease. Up to date, it has been many researchers try to find effective ways to control bacterial diseases. Recent studies indicated that synbiotics induced penaeid shrimp immunity and promoted the growth of aquatic animals (reviewed in Huynh et al., 2017), and oxytetracycline was a highly effective cure for spiroplasma disease (Feng et al. 2010). Some immuno-related genes or proteins, such as tachylectin-like genes and proteins (Angthong et al. 2017), heat shock proteins (Junprung et al. 2017), etc., have been verified that were involved in shimp tolerance to AHPND-causing strain. Crustacean fibrinogen-related proteins were involved in innate immune response during the AHPND or other pathogens infections (Angthong et al. 2017). In addition, viruses remain a major obstacle to crustacean aquaculture. Recent research has shown that some new and newly emerging diseases in shrimp, including hepatopancreatic microsporidiosis, hepatopancreatic haplosporidiosis, aggregated transformed microvilli, covert mortality disease, white spot disease, yellow head disease, infectious myonecrosis, and white tail disease, etc. turn into as the most serious viral threats to commercially exploited shrimps (Thitamadee et al. 2016).

10.1.7.2 Temperature, Desiccation and Hypoxia/Anoxia Stress

It is well known that temperature, as one of abiotic factors, has the potential to alter physiology (Tomanek 2010; Somero 2012; Barber et al. 2016), behavior (Huey 1991), and species interactions (Morley and Lewis, 2014; Labaude et al., 2017a). Thermal fluctuations have been considered as one of the most important factors that affect the integrity of physiological system at various cellular and molecular levels (Cossins and Bowler 1987). Temperature affects molecular and physiological processes and impacts the organism's activity pattern (Montagna 2011; Habashy and Hassan 2010). When cells perceived acute ambient temperature fluctuations, aquatic organisms may manifest physiological responses to punctuated temperature spikes long before behavioral responses (Bedulina et al. 2017). Studies on marine species have shown that the thermal tolerance limits are determined by the onset of hypoxaemia, resulted in the activation of anaerobic metabolism pathways (Pörtner 2002). According to Paine (1974), temperature and desiccation are amongst the most

relevant factors in rocky shores, setting the upper limits of the species' distribution. Extreme desiccation stress can also causing the eggs diapause of crustaceans. However, organisms possess adaptive mechanism, such as thermal tolerance, heat-shock protein expression and protein thermal stability, etc., to response against environmental extremes and decrease cell damage. The cellular stress response is activated to maintain cellular functioning and improves the organism's ability to cope with certain situations (Morris et al. 2013). This response consists in the activation of cellular pathways such as proteolysis through the ubiquitin-proteasome pathway and the increased production of heat shock proteins (Madeira et al. 2015).

10.1.7.3 Osmotic Stress

Osmotic stress is the most common environmental stress factor for aquatic organisms. Osmoregulation plays an important role being one of the most important regulatory functions in aquatic organisms to maintain osmotic homeostasis. Several studies (Charmantier 1975; Burton 1986) have also reported the influence of stressors as temperature or salinity on organisms by altering the osmoregulation capability via elevated levels of Na^+-K^+ ATPase activity (Charmantier 1998) or heat shock protein (Sun et al. 2012; Madeira et al. 2014) to sustain relative osmotic haemolymph homeostasis (Romano and Zeng 2006). To date, several studies have reported the expression profiles of HSP under salinity stress. For instance, the expression of HSP90 was induced when *Crassostrea hongkongensis* (Fu et al. 2011) and *Eriocheir sinensis* (Li et al., 2009b; Sun et al. 2012) were subject to osmotic stress. HSP70 in the hemocytes of *Scylla paramamosain* was significant increasing expression after high salinity stress (Yang et al. 2013). HSP60, HSP70 and HSP90 in the hepatopancreas of the swimming crabs *Portunus trituberculatus* exhibited downregulated or upregulated expression profiles exposed to low salinity (4 ppt) (Bao et al. 2014). Thus, HSP were deemed participate in mediating the salinity stress in aquatic crustaceans.

10.1.7.4 Ultraviolet Radiation Stress

Ultraviolet (UV) radiation is one of abiotic factors and can cause direct or indirect damage to organisms. UV radiation can directly cause alterations in protein synthesis and DNA through absorption of high-energy photons and can indirectly generate reactive oxygen species, which cause diverse damage to proteins, nucleic acids, and lipids (Kim et al., 2011b; Rhee et al. 2012; Won et al. 2014). The effects of ultraviolet (UV) radiation on aquatic organisms have been a great concern in recent years. Study on calanoid copepod has demonstrated that UV-induced stress depresses the feeding mechanisms, digestion, and affects the entire food chain (Lacuna and Uye, 2001). UV radiation directly and indirectly affects survival, growth, and reproduction, and increased expression of antioxidant enzymes and HSP genes in the copepod *Paracyclopina nana* (Won et al. 2014; Won et al. 2015).

10.1.7.5 Heavy Metals Stress

Heavy metals are among the most problematic causes of water, soil and plant pollution. They enter waters through seepage from household or industrial waste. Currently, heavy metal pollution becomes a serious problem in aquatic ecosystems for the aquaculture animals. Numerous ecotoxicological laboratory studies on crustaceans have focused on heavy metal-induced gene expression changes. Copper (Cu), silver (Ag), zinc (Zn), plumbum (Pb), manganese (Mn), arsenic (As) and cadmium (Cd) are the most common heavy metals tested in these studies (Yamuna et al. 2000; Timofeev et al. 2008; Rhee et al. 2009; Luan et al. 2010; Kim et al. 2011a; Qian et al. 2012; Kim et al. 2014; Pestana et al. 2016). Heavy metal stress is also closely associated with the induction of oxidative stress. Heavy metals in seawater can cause oxidative stress in various living organisms including the marine crab *Portunus trituberculatus* (Zhang et al. 2009). Oxidative stress can induces a cellular redox imbalance and protective stress response. Many studies on aquatic organisms, especially in crustaceans, have demonstrated that heavy metal stress can significant induce the synthesis of antioxidant enzymes (Kim et al., 2011a) or heat shock proteins (Rhee et al. 2009; Kim et al. 2014; Pestana et al. 2016). HSP seem to play important role in the intrinsic immune system and stress responses of crustaceans (Qian et al. 2012; Huang et al. 2011; Kim et al. 2014; Pestana et al. 2016).

10.1.7.6 Endocrine Disruptor Chemicals

Endocrine disruptor chemicals (EDCs) are compounds that mimic natural hormones inhibiting their activity or altering their normal regulatory function within the immune, nervous, and endocrine systems (Lauritano et al. 2012). These chemicals are of ecotoxicological importance due to their tendency to be absorbed onto humic material or into aquatic organisms, to accumulate, and to persist in water or food web for a long time. Thus, their effects can cause a long period stress to aquatic organisms. To date, several EDCs, such as pesticides, bisphenol A, phthalates, dioxins, and phytoestrogens, can interact with the female reproductive system and lead to endocrine disruption (Costa et al. 2014). Endosulfan and deltamethrin are commonly used pesticides in shrimp farms (Tu et al. 2007). Moreover, endosulfan is often used as a broad-spectrum insecticide mainly in agriculture. Insecticide is considered to be highly toxic to aquatic organisms (Lombardi et al. 2001; Wirth et al. 2001, 2002; Capkin et al. 2006). Studies on EDCs induced stress response indicated that HSP family proteins (Seo et al. 2006; Rhee et al. 2009; Dorts et al. 2009; Cocci et al. 2017), detoxification enzymes glutathione S-transferases (Lee et al. 2006) and superoxide dismutase (Kim et al., 2011a) were significant induced, and these proteins are considered potentially involved in aquatic organisms protection against stress.

10.1.7.7 Other Toxicants

In addition to the above-mentioned main chemicals, there are a large number of other toxicants in the habitats of aquatic organisms. These toxicants including hydrocarbons, diatom toxins, emamectin benzoate, nitrite, and prooxidant chemical hydrogen peroxide (H_2O_2), etc. They are accumulated into aquatic and/or terrestrial environment via the discharge of household and/or industrial waste. Studies have demonstrated that these toxicants can induce toxic effects on crustaceans (Ianora et al. 2004; Hansen et al. 2008; Hansen et al. 2011). Lauritano et al. (2011) has shown that two days of feeding on a strong oxylipin-producing diatom (*Skeletonema marinoi*) resulted in strongly down-regulated heat shock proteins (HSP40 and HSP70) in the crustacean copepod *Calanus helgolandicus*. Diatom oxylipins are also known can induce an increase in free radicals, such as reactive oxygen species that can induce oxidative stress and damage to cell (Fontana et al. 2007). Additionally, nitrite is considered as one of the most common pollutants in aquaculture because of its multiple integrated effects. Study on shrimp has demonstrated oxidative stress was one of the toxicity mechanisms of nitrite (Xian et al. 2011). Guo et al. (2013) has verified that nitrite exposure induces expression of apoptosis-related genes in hemocytes, meanwhile, expression levels of HSP70 and antioxidant enzymes up-regulated to protect the hemocyte against nitrite stress.

10.1.8 Heat Shock Proteins in Aquaculture Disease Immunology and Stress Response

Heat shock proteins have been first described in *Drosophila busckii* in response to stress (Ritossa 1962). Interest in the putative roles of HSP has been intensified since their identification as chaperones protecting cellular proteins against denaturation (Sanders 1993; Feder and Hofmman, 1999). Further studies have focused on the HSP of aquaculture animals due to their importance in coping with stress-induced denaturation of client proteins, as well as their essential roles including folding, assembly, degradation of other proteins, and the regulation of gene expression (Piano et al. 2005; Terasawa et al. 2005). The synthesis of HSP can be induced by physiological and environmental stress conditions, such as high thermal shock, heavy metals, free radicals, desiccation, microbial infection and other stressors. The induction of HSP is considered as an important protective, ecophysiologically adaptive, and genetically conserved response to environmental stress in all organisms. In recent years, researchers have investigated that the heat shock chaperonin are involved in autoimmune and innate immune response in several species including crustaceans, and HSP plays protective immune response against many bacterial and viral diseases (Habich and Burkart 2007; Li et al. 2011; Sung and MacRae 2011; Chaurasia et al. 2016). Currently, considering the enormous economic losses induced by environmental stressors in the crustacean aquaculture industry, studies focusing on heat shock proteins have become increasingly popular because of the

important roles that these proteins participate in resistance to various stressors (Huang et al. 2008; Rhee et al. 2009; Qian et al. 2012; Kim et al. 2014; Won et al. 2015; Chaurasia et al. 2016; Shi et al. 2016; Bedulina et al. 2017; Junprung et al. 2017; Henry et al. 2017). Extensive studies have been carried out to investigate the structure and functions of HSP, cross-talks and immune response mechanisms, and innate immune pathways in crustaceans after were exposed to various environmental stressors or xenobiotics. Exploiting HSP for prevention and treatment of aquaculture diseases in commercially cultured aquatic organisms is important because it offers an alternative to the utilization of antibiotics and therapeutic drugs (Sung and MacRae 2011). Moreover, previous studies have attempted to find effective strategies for dealing with environmental stressor in aquaculture of aquatic organisms.

10.1.8.1 The Structure and Functions of HSP

HSP, known as molecular chaperones or chaperonins (Ellis 1987), play a crucial role in productive folding and assembly of cellular proteins into highly ordered oligomeric structures. In eukaryotes, HSP are categorized into several families (Fig. 10.1) and many members of HSP families have counterparts, referred to as "constitutive chaperones" or "heat shock cognates" (HSCs). HSCs play a fundamental role in the regulation of normal protein synthesis within the cell. Each HSP family has unique structural and functional features that affect substrate preference and co-chaperone interactions (see review Bar-Lavan et al. 2016). Except for sHSP, members of other HSP families require ATP hydrolysis for their function, which is via specific ATPase domain to bind proteins undergoing denaturation, prevent their misfolding and drive allosteric rearrangement of misfolding proteins (Kahn et al., 1993). Nonetheless, in the process of aiding nascent polypeptide folding and oligomerization, protecting proteins from irreversible denaturation, re-folding or degrading damaged proteins, different HSP families have separately regulated chaperone networks (Fig. 10.1). And even the expression of components of the chaperone network is tissue-specific in the activation of the heat shock response (Guisbert et al. 2013).

The HSP function cooperatively by forming intracellular networks of chaperones, co-chaperones and accessory proteins. For example, a bi-chaperone system composed of HSP70 and HSP100 chaperones in plants to rescue aggregated proteins and provide thermotolerance to cells (Mogk et al. 2015). HSP100 proteins bind to the ATPase domain of HSP70 via their unique M-domain. HSP70 has a conserved nucleotide-terminal ATP binding domain (NBD) connected by a highly conserved hydrophobic flexible linker to a variable, carboxyl-terminal substrate binding domain (SBD) (Schlecht et al. 2011). Members of the HSP70 family form a central hub of the chaperone network controlling alla spects of proteostasis in the ATP-containing compartments of eukaryotic cells (Finka et al. 2015). HSP70s can function as ATP-fuelled unfolding nanomachines capable of switching polypeptides between different folded states. Under stress conditions, HSP70s can bind and prevent the aggregation of misfolding proteins and thereafter act alone or in collaboration with other unfolding chaperones to solubilize protein aggregates

(Finka et al. 2015). Based on this type of cooperative mechanism among different chaperones, suppositional explanations in literatures about intracellular accumulation of HSP70 protects against protein damage upon viral and bacterial challenge or toxicant stress are intelligible. Additionally, it has been reported that HSP70s together with HSP90s can control the activation of the steroid hormone receptors (Dittmar et al. 1998). HSP90 is composed of monomers with three domains. The highly conserved N-terminal domain contains ATP-binding and hydrolysis site, the middle region interacts with substrates and co-chaperones, and the C-terminal domain possessing a MEEVD motif for binding co-chaperones with tetratricopeptide repeats (Hagn et al. 2011; Bar-Lavan et al. 2016). HSP90 forms a hub that controls numerous important signalling pathways in eukaryotic cells (Taipale et al. 2010; Taipale et al. 2014). It act at later stages of substrate folding, binding to partially folded intermediates. HSP90 can also forms complex with HSP70/HSP40 associated proteins and co-chaperones HSP70/HSP90 organizing protein (Taipale et al. 2010; Bar-Lavan et al. 2016). Currently, it is clear that HSP70 function can be regulated by co-chaperones. HSP40, act as co-chaperone of HSP70, deliver the substrate to HSP70 and stimulate the ATP hydrolysing activity of the chaperone (Bar-Lavan et al. 2016).

HSP60 is a multi-functional immunogenic chaperone, composed of two rings positioned back-to-back or a toroid quaternary structure that constructed with different monomers (Liang and MacRae 1997; Yébenes et al. 2011). Some researchers have reported HSP60 is involved in a variety of autoimmune and inflammatory processes (Shi et al. 2016; Chaurasia et al. 2016; Sathyamoorthy et al. 2017). HSP60 has the ability to refold proteins denatured during stress and it may interact with peptides and proteins involved in the invertebrate immune response (Sung and MacRae 2011). Moreover, HSP60 can be expressed in response to various external stressors such as osmotic stress, toxic metals exposure, and pathogenic infections (Shi et al. 2016; Chaurasia et al. 2016). HSP60/HSP10 may be combined to produce a chaperone complex with effective chaperone and ATPase activities (Shi et al. 2016). In addition, members of sHSP chaperone family have also the ability to bind proteins undergoing denaturation and prevent their misfolding. sHSP monomers, consisting of a conserved α-crystallin domain flanked by a N-terminal sequence and a C-terminal extension (Sun and MacRae 2005; Mchaourab et al. 2009; Hilario et al. 2011), assemble into oligomers (Kennaway et al. 2005; Laganowsky et al. 2010; Jehle et al. 2011). sHSP oligomers either disassemble or undergo structural rearrangement during stress conditions, to provide a large surface area that can bind and sequester aggregation-sensitive folding intermediates and prevent their aggregation (Suss and Reichmann 2015; Bar-Lavan et al. 2016). To date, it is clear that sHSP conformational plasticity is required for their function (Suss and Reichmann 2015). Proteins released from sHSP when stress passes either refold spontaneously or with the assistance of ATP-dependent chaperone systems, such as HSP70, alone or in concert with HSP100 (Ehrnsperger et al. 1997; Lee and Vierling 2000; Haslbeck and Vierling 2015).

10.1.8.2 HSP and Stress Response in Crustaceans

Due to the stimulation under different stressful situations, heat shock proteins are collectively known as stress proteins or stress biomarker. Highly conserved and ubiquitous across species, most HSP are expressed strictly in response to physiological or environmental stress, play important roles in the defense against pH challenge, osmotic stress, heavy metal stress, and pathogenic infection, etc. (Shi et al. 2016). In crustaceans, when they exposed to various abiotic or chemical stresses, such as heat shock, cold shock, oxidation, ultraviolet radiation, hypoxia, bacterial and viral infections, nutrient deprivation, toxins, and malignant agents, etc. One physiological stress where HSP induction has been shown to play a significant role is in relation to osmotic shock. A broad spectrum of aquatic animals has been shown to react to osmotic stress through HSP production, particularly HSP70. Studies have shown that members of the HSP70 family are stress-inducible and constitutive proteins that play essential roles in protein metabolism under both normal and stress conditions, including de novo protein folding, membrane translocation, degradation of misfolded proteins and other regulatory processes (Multhoff 2007; Ranford and Henderson 2002). HSP70 expression of *Tigriopus japonicus* is modulated by environmental toxicants at low concentration ranges (Rhee et al. 2009). More recently studies demonstrated that HSP70 production was impaired in crabs *Pachygrapsus marmoratus* were short-term exposured to thermal ramp at standard pH (8) and hyposaline conditions (15‰), or to thermal ramp at lower pH (7) and standard saline conditions (35‰). Temperature increase has decreased osmolality of *Pachygrapsus marmoratus*. In addition, the expression of HSP70 and HSP90 genes in the lobster *Homarus americanus* (Spees et al. 2002) and in juvenile mitten crabs *Eriocheir sinensis* (Sun et al. 2012) exhibits major changes under both low and high salinities. Other study has indicated that HSP genes (HSP60, HSP70, and HSP90) are involved in the adaptation of *Eriocheir sinensis* to low salinity exposure, and that different HSP have diverse functions in response to low salinity stress (Bao et al. 2014).

Some researchers have put forward the combination of multiple stressors can result in various interactions. Several studies have demonstrated up-regulation of HSP70 in response to a wide array of stressors, including thermal and NH_3 stress, in a variety of arthropod taxa (Feder and Hofmann 1999; Sung et al. 2014). Recently Côté et al. (2016) defined these patterns in relation to the neutral additive interaction in which the effect of multiple stress is equal to the sum of each isolated stress. However, study on combined effect of temperature and ammonia on crustacean *Gammarus pulex* have demonstrated that the effects of combined environmental stressors was strongly differ from simple additive effects, NH_3 concentration had no effect on HSP70 mRNA synthesis across temperatures (Henry et al. 2017). Further investigations should be conducted to conclude more generally reliable conclusions.

Pollution and temperature are two of the most important stressors that aquatic organisms are facing. Numerous laboratory experiments and field investigations

have demonstrated that the relationship between exposure to stressors such as heavy metals or environmental toxins and synthesis of HSP has the potential to act as a significant indicator of environmental stress. The effects of stress induced by toxicants and temperature can be assessed via different heat shock proteins as stress markers. However, before choosing monitoring specific objects, it is necessary to perform preliminary studies to reveal possible deviations of parameters of interest from the standard values (Timofeev et al. 2008). For example, study on endemic Baikal amphipods has indicated that temperature- and toxicant-induced stresses induced sHSP synthesis in this species has been recorded, while induction of HSP70 synthesis in the same species after temperature stress has not been detected (Timofeev et al. 2008). So, HSP can be used as stress markers in Baikal species. Nevertheless, other study on the copepods has demonstrated that the *T. japonicus* HSP70 transcript is a strong biomarker towards diverse environmental stressors including heavy metals (Rhee et al. 2009). Kim et al. (2014) has extensively discussed the ecotoxicological aspects of HSP synthesis and their potential as an environmental monitoring tool of heavy metal pollution. Results indicated HSP genes such as HSP20 and HSP70 are useful as potential biomarkers in the monitoring of heavy metals. In addition, HSP70 in *Oniscus asellus* has shown that peak of HSP70 response after 24 h of exposure and a second peak after several days of exposure to organic chemicals γ-hexachlorocyclohexane (γ-HCH) and/or pentachlorophenol (PCP) (Köhler et al., 1999). HSP70 can act as a biomarker of chronic exposure and effect for PCP and γ-HCH. Chang (2005) has examined the influence of heat-shock, osmotic stress, and the molt cycle upon HSP (HSP70 and HSP90) expression at the protein and mRNA levels in lobsters *Homarus americanus* and *Nephrops norvegicus*. Results has demonstrated tissue-specific HSP responses and quantification of these different stress responses were suggested might serve as early indicators of the degradation of environmental health (Chang 2005). Study on Chinese shrimp *Fenneropenaeus chinensis* under different stresses has revealed that heat shock treatment induced up-regulation of HSP70 and HSC70, and cadmium treatment caused down-regulation of HSC70. Therefore, HSP70 or HSC70 could be developed as a biomarker to indicate different stresses in shrimp (Luan et al. 2010). Further study on the pacific white shrimp *Litopenaeus vannamei* has shown that HSP70 was sensitive to temperature fluctuations, iron and zinc stress, while HSP60 displayed particularly high sensitivity to cadmium and manganese exposure (Qian et al. 2012). HSP70 may be most suitable to act as a biomarker indicating thermal stress, iron and zinc stimulation, while HSP60 may be a promising candidate marker of pH stress, cadmium and manganese exposure in shrimp. Similarly, study on mud crab *Scylla serrata* has revealed HSP70 was significant induced under different stress conditions, HSP70 could be used as a biomarker in environmental monitoring (Fu et al. 2013).

Several aspects of the stress response have been examined as possible biomarkers for the measurement of sublethal toxicity. For instance, the effects of sublethal concentrations of six different endocrine disruptor chemicals on HSP20 gene expression of *T. japonicus* were examined (Seo et al. 2006) and indicated that

4–40-octylphenol (OP) and 4-nonylphenol (NP) induced a dose-dependent increase. Moreover, benzo[α]pyrene induced a significant decrease at 25 mg/L of HSP20 mRNA levels and an increase at higher doses (50 and 100 mg/L). Further test (Rhee et al. 2009) in the same species has demonstrated that HSP70 was induced significant down-regulation under NP and OP exposure at each concentration, while a significant increase in HSP70 gene expression was found at 1,10 and 20 mg/L bisphenol A. Furthermore, the effects of HSP in the black tiger shrimp *Penaeus monodon* exposure to deltamethrin and/or endosulfan were assessed, results have shown that HSP90 level induced up to approximately 2.1-fold increase. Therefore, HSP90 levels may be potential discriminating biomarkers to assess the chemical stress level in farm shrimp (Dorts et al. 2009). In addition, recently study has also demonstrated that non-lethal heat shock increases tolerance to metal exposure in brine shrimp (Pestana et al. 2016).

10.1.8.3 HSP and Aquaculture Disease Immunity

Infectious diseases especially new emerging diseases have caused enormous economic loss in crustacean aquaculture industry in recent years (Thitamadee et al. 2016). Crustaceans have an innate immune system and it convene some metabolic enzymes (e.g. cytochrome P450, glutathione S-transferase, superoxide dismutase, etc.), heat shock proteins, hemocyte- and plasma-derived immune factors, and immune-related proteins, etc. in crustaceans to enhance disease tolerance (Cerenius et al. 2010; Lauritano et al. 2012; Junprung et al. 2017). It is well recognized that a non-lethal heat shock will activate the transcription and synthesis of HSP, which protect against subsequent stressors. HSP70 accumulation induced by abiotic stress in *Artemia* larvae was associated with enhanced resistance to infection by pathogenic *Vibrio campbellii*, perhaps via stimulation of the *Artemia* immune system (Sung et al. 2008). In a study (Huang et al. 2008), researchers have investigated the effects of white spot syndrome virus (WSSV) infection on gene expression in shrimp *Penaeus monodon*, results showed that an HSP21 gene was highly expressed in all organs but down-regulated with WSSV infection, suggested that regulation of this gene, and hence its product was seriously inhibited by WSSV. In another study, the expression levels of HSP21, HSP70 and HSP90 in *P. monodon* were significantly induced increase after *Vibrio harveyi* injection. This evidence suggested HSP genes participate in immunity response against pathogenic infection (Rungrassamee et al. 2010). Moreover, the expression level of HSP60 was significantly up-regulated in the gills, hepatopancreas and haemocytes of white shrimp *Litopenaeus vannamei*, while HSP70 transcripts were also induced in haemocytes and the hepatopancreas after the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Vibrio alginolyticus*. Results has indicated that HSP participate in mediating the immune responses of the white shrimp to bacterial challenge, and the calcium ion signaling transduction pathway may be involved in the initiation of the shrimp's immune responses in early stages of infection (Zhou et al. 2010).

To date, HSP activity in relation to pathogenesis in crustaceans has been studied in detail (Sung and MacRae 2011). Vast literatures have verified that different members of HSP families were involved in immune responses against various pathogens challenge and diseases in crustaceans. For instance, HSP37 in freshwater prawn *Macrobrachium rosenbergii* was potentially involved in the immune responses against hypodermal and hematopoietic necrosis virus challenge (Arockiaraj et al. 2012). Study on mud crab *Scylla serrata* has revealed HSP70 was significant induced under different stress conditions, it could be used as a disease resistance factor used in application (Fu et al. 2013). Recent study (Chaurasia et al. 2016) has suggested that HSP60, 70 and 90 molecules can protecting cells against pathogens as well as severe cellular and environmental stresses in crustaceans. Additionally, recent experimental studies by Junprung and co-workers have investigated whether non-lethal heat shock (NLHS) could enhance the survival of shrimp *Penaeus vannamei* upon challenge with an acute hepatopancreatic necrosis disease (AHPND) causing strain of *Vibrio Parahaemolyticus* (VP_{AHPND}), revealed that the expression of HSP70 and HSP90 were induced upon exposure of shrimp to chronic NLHS. Results suggested that HSP played crucial roles in bacterial defense in shrimp and the NLHS enhance the shrimp tolerance to VP_{AHPND} infection (Junprung et al. 2017). Moreover, these investigations contributed significantly to understanding of the significance of HSP in crustacean pathology immunity.

10.1.8.4 Exogenous HSP Stimulation

The application of exogenous HSP stimulation in relation to stress- or cross-tolerance in crustaceans are limited. Previous studies have shown that feeding brine shrimp *Artemia franciscana* (Kellogg) larvae with *Escherichia coli* which had been previously heat-shocked induced them to overproduce different prokaryotic HSP (Sung et al. 2009a; Sung et al. 2009b). Moreover, up-regulation of endogenous HSP70 in brine shrimps were concurrent with enhanced resistance to *Vibrio* infection, results in enhances survival approximately two- to three-fold upon challenge with pathogenic *Vibrio campbellii*. These works supplied a potential novel strategy to control pathogenic infection and disease in aquatic organisms. To verify this, Baruah and co-workers have performed a series of experimental studies in recent years. Feeding DnaK-enriched bacteria stimulates the prophenoloxidase (ProPO) cascade system of *Artemia* offered an immunological effect against *Vibrio* infection (Baruah et al. 2010; Baruah et al. 2011). Then, the function of exogenously patented extract Pro-Tex® (a soluble version of Tex-OE®) as a novel therapeutant has been further examined in cultured brine shrimp *Artemia franciscana*. Results has demonstrated that Tex-OE® enhanced HSP70 expression in *Artemia* and can function in synergy with a non-lethal heat shock to protect *Artemia* against abiotic stressors (Baruah et al. 2012). Tex-OE® could be a potential candidate for use as an anti-stressor in aquaculture (Roberts et al. 2010; Baruah et al. 2012; Baruah et al. 2014). Additionally, study of Baruah et al. (2013) has demonstrated that cultured *Artemia*

fed with recombinant C-terminal fragment of *Artemia* HSP70 or DnaK protein were well protected against virulent *Vibrio* challenge, and the prophenoloxidase system was also markedly induced by these truncated proteins.

Taken together, these studies indicated feeding aquatic organisms with HSP-enriched bacteria or commercial anti-stress agents is an effective approach to assist disease control in crustacean aquaculture.

10.2 Conclusions and Future Prospects

Heat shock proteins participate in environmental stress responses in aquatic organisms exposed to various stressors, such as temperature, UV irradiation, salinity, hypoxia, pathogenic infection, heavy metals or other toxic chemicals, etc. Increasing HSP synthesis in aquatic organisms by heat shock, chemical application and feeding exogenous HSP also enhances resistance to infection. The level of tolerance are associated with the accumulation of HSP in vivo. HSP as agents of disease resistance and an excellent possibility that their application (e.g. the application of pathogen-derived HSP vaccines) will present a significant advance in preventing diseases of aquatic organisms used for aquaculture in future.

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Part IV
Parasites

Chapter 11
Heat Shock Proteins in Parasitic Flatworms

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Abstract During the entire life cycle, parasitic flatworms experience great changes in growth environment in aspects of temperature, nutrient, pH, and immune responses. They have evolved to have multiple mechanisms to survive these stresses, and one is to orchestrate expression of heat shock proteins (HSP), a conserved protein family that plays a crucial role in maintenance of protein homeostasis. These parasites encode a considerable number of *hsp* genes, some which lack typical domains/motifs or are expressed in a secretory pattern via multiple secretory pathways. Flatworm HSP has been shown to offer protection from stress-induced damages as well as to be immunogenic and immunomodulative, rendering them to play a role in parasite development and modulation of immune responses. Due to the immunogenic and immunomodulative properties, some flatworm HSP have been demonstrated to be promising diagnostic and vaccine candidates. Using nanotechnology, development of effective HSP-targeting antagonists is of high veterinary interest. It will allow us to use these small molecules to pinpoint a role of flatworm HSP during parasitism, which in turn aids us to better control parasite transmission.

Keywords Diagnosis · Drug target · Flatworms · HSP · Immunogenicity · Secretion · Vaccine

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Abbreviations

ESP	Excretory/secretory products
EVs	Extracellular vesicles
HSF1	Heat-shock transcription factor 1
HSP	Heat shock proteins
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription PCR
sHSP	Small heat shock proteins
TLRs	Toll-like receptors
Treg cells	Regulatory T cells

11.1 Introduction

The Phylum Platyhelminthes is composed of free living Turbellaria and strictly parasitic Cestoda, Monogenea, and Trematoda, with estimated species of 20,000. Of them, although their evolutionary relationship is still controversial (Egger et al. 2015; Fromm et al. 2013), parasitic platyhelminthes (flatworms) all generally need two or more different hosts to develop and complete the life cycle, some of which are of medical and/or veterinary significance, such as *Schistosoma japonicum* responsible for schistosomiasis, *Taenia solium* for cysticercosis, and *Echinococcus granulosus* for cystic echinococcosis. During the entire life cycle, parasites constantly experience great shifts in growth environment in aspects of temperature, nutrient, pH, and immune responses. To cope with harsh conditions, they have evolved multiple effective strategies to facilitate development and prevent clearance by a host. In addition to alteration of parasite surface components, regulation of host immune cells' functions, and amelioration of growth microenvironment (Zheng 2013), another mechanism is to initiate stress responses by dynamic modulation of expression of heat shock proteins (HSP) (Perez-Morales and Espinoza 2015).

HSP is a large protein family that, as a molecular chaperone, is primarily involved in acceleration of protein proper folding and plays an important role in maintenance of protein homeostasis in a cell. Directly based on molecular weight and sequence homology, HSP members are grouped into several subfamilies, including HSP110, HSP100, HSP90, HSP70, HSP60, HSP10, and small HSP (sHSP). Glucose-regulated proteins are also a class of HSP, which are localized in endoplasmic reticulum. Compared with other subfamilies, sHSP members share much lower sequence identity, generally being no more than 50% (Perez-Morales and Espinoza 2015). Despite the high sequence divergence, sHSP form conserved secondary and tertiary structures. sHSP are structurally characterized by a highly conserved α–crystallin domain, which is usually located at a carboxyl terminus. Two less conserved domains, N- and C-terminal domains, are also found in sHSP. All the three domains are essential for sHSP oligomerization and functions (Carra et al. 2017). Similarly, all the other HSP subfamily members also have several known conserved domains

in a given HSP. For example, HSP70 comprises an N-terminal domain with ATPase activity, a C-terminal domain with peptide-binding capacity, and a conserved EEVD motif at the far carboxyl terminus. Some of these domains are also present in other HSP, such as HSP90 (Bolhassani and Rafati 2008; Jee 2016). Currently, non-canonical cytosolic HSP70s without the EEVD motif were described in cestodes (Tsai et al. 2013).

In addition to heat shock, other stresses such as ions, pH, and reactive oxygen molecules are also potent regulators of HSP expression. It is noted that expression of most *hsp* genes is inducible, but some are clearly expressed in nature (Feder and Hofmann 1999). It is well documented that, although their expression is commonly orchestrated by heat-induced heat shock factors, different families show remarkable differences in distributions and functions within a given cell (Jee 2016). Most HSP primarily act as a molecule chaperone to bind to target clients and regulate their folding, assembly, and decay. Additionally, HSP is involved in many other biological processes and closely linked to occurrence of a number of diseases (Bolhassani and Rafati 2008; Carra et al. 2017). In the course of parasitic infections, expression of *hsp* genes is tightly regulated and HSP is considered as an immunodominant antigen, involved in immune responses, parasite virulence and transmission.

11.1.1 Overview of HSP in Parasitic Flatworms

With the availability of complete genomes, genome-wide identification of HSP is possible. In general, current data indicate that all the parasitic flatworms sequenced have a considerable amount of *hsp* genes (Table 11.1), especially some *Taenia* and *Echinococcus* species. Independent and parallel expansions of HSP110 and cytosolic HSP70 were observed in these cestodes, and the gene copy number of cytosolic HSP70 was expanded to 22 in *Echinococcus* species and to 32 in *Taenia* species (Tsai et al. 2013; Wang et al. 2016a). Interestingly, substantial numbers of *hsp70* genes are positioned in the subtelomeric regions in *Echinococcus multilocularis* (Tsai et al. 2013), a tiny tapeworm responsible for alveolar echinococcosis in human beings. Such genomic arrangements and biological functions of these *hsp70* genes are still open questions. Moreover, *Echinococcus* species possess cytoplasmic *hsp70*-like pseudogenes with several copies in the genomes, which might have originated from successive duplications of one copy. It was further shown that these pseudogenes were expressed in protoscoleces and adult worms of *E. granulosus* (Koziol et al. 2009), but their role remains elusive. In response to anthelmintic treatments, HSP exhibits divergence in an expression pattern. Ivermetin alone or combined with albendazole downregulated the HSP70 level, and only ivermetin alone upregulated the HSP60 level in treated *E. granulosus* protoscoleces. On the contrary, praziquantel alone or combined with albendazole had no effects on the HSP70 expression (Martinez et al. 1999). Although HSP tends to be inducible and is differentially expressed during the life cycle, it is becoming clear that some HSP are constantly expressed throughout the entire developmental stages of tapeworms. For instance, expression of HSP20 homologous to *Schistosoma mansoni* Sm-p40 occurs

Table 11.1 HSP in parasitic flatworms

Species	Number[a]	Representative[b]	Expression	Function	Reference
Cestoda					
Echinococcus canadensis	18/16	/[c]	/	/	
Echinococcus granulosus	57/0	/	/	/	
Echinococcus multilocularis	59/14	HSP20 (sm-p40-like)	Oncosphere, metacestode, adult worm	Stimulate immune responses	Huang et al. (2016), Kouguchi et al. 2010, Merckelbach et al. (2003), Tsai et al. (2013)
		HSP70	Oncosphere, metacestode	Stimulate immune responses	Huang et al. (2016)
Hydatigera taeniaeformis	20/5	/	/	/	
Hymenolepis diminuta	21/9	/	/	/	
Hymenolepis microstoma	15/4	/	/	/	
Hymenolepis nana	23/11	/	/	/	
Taenia asiatica	112/56	/	/	/	
Taenia saginata	179/179	/	/	/	
Taenia solium	81/44	/	/	/	
Diphyllobothrium latum	24/11	/	/	/	
Mesocestoides corti	24/6	/	/	/	
Schistocephalus solidus	23/11	/	/	/	
Spirometra erinaceieuropaei	44/24	/	/	/	
Trematoda					
Clonorchis sinensis	15/0	HSP70	Adult worm	Induce a Th1 response	Chung et al. (2017)
		HSP90	Adult worm	Induce a Th1 response	Chung et al. (2017)
Echinostoma caproni	46/32	/	/	/	
Fasciola hepatica	45/26	/	/	/	
Opisthorchis viverrini	28/26	/	/	/	

(continued)

Table 11.1 (continued)

Species	Number[a]	Representative[b]	Expression	Function	Reference
Schistosoma curassoni	19/3	/	/	/	
Schistosoma haematobium	17/9	/	/	/	
Schistosoma japonicum	11/4	HSP60	Egg, adult worm	Induce CD4+CD25+ T cell development	Wang et al. (2009a)
		Mortalin-like HSP70	Egg, cercaria, schistosomula, adult worm	Protect from heat stress, induce a Th1 immune response	He et al. (2010)
		sj648/HSP70	Egg, cercaria, schistosomula, adult worm	Skew towards a Th2-type immune response	Yang et al. (2012)
		HSP90, phosphorylated	Schistosomula, adult worm	Participate schistosome development possibly by interacting with other signal molecules	Cheng et al. (2013)
Schistosoma mansoni	20/2	Sm-p40	Egg, miracidia	Protect from extreme physiological stresses	Sotillo et al. (2015)
		HSP70	Egg, miracidia, schistosomula	Participate protein maturation, stabilize macromolecular structure, transport lipids and/or lipoprotein, protect from immune attacks	Cass et al. (2007), Mathieson and Wilson (2010), Pereira et al. (2015), Sotillo et al. (2015)
Schistosoma margrebowiei	19/11	/	/	/	
Schistosoma mattheei	18/8	/	/	/	
Schistosoma rodhaini	19/8	/	/	/	
Trichobilharzia regenti	27/10	/	/	/	

(continued)

Table 11.1 (continued)

Species	Number[a]	Representative[b]	Expression	Function	Reference
Monogenea					
Gyrodactylus salaris	35/35	/	/	/	
Protopolystoma xenopodis	29/17	/	/	/	

[a]Gene copy numbers of *hsp* genes in parasitic flatworms. One number before slash represents the total copy number of *hsp* genes in a corresponding parasite, and the other after slash represents the number of *hsp* genes that are not fully annotated in the database. As these unannotated genes contain HSP-specific domain(s), they are considered as potential *hsp* genes and counted into the total copy number. Therefore, the total copy number of *hsp* genes in all the parasitic flatworms is not final and is updated with improvement of genome annotation. Data are from http://parasite.wormbase.org/species.html.
[b]HSP well studied.
[c]'/': not applicable.

in dormant and activated oncospheres, metacestodes, and adult worms of *E. multilocularis* (Huang et al. 2016; Merckelbach et al. 2003; Tsai et al. 2013).

The copy number of *hsp* genes is various in trematodes. Compared with *Taenia* species, most flukes including *Schistosoma* species and *Clonorchis sinensis* have much less *hsp* genes, with a total gene copy number being no more than 20 (Table 11.1). Despite a relatively lower gene copy number, a comparative proteomic analysis revealed that HSP was the most abundant proteins in immature and mature eggs as well as miracidia of *S. mansoni*, accounting for more than 20% of total proteins, but different HSP showed distinct abundance patterns. In particular, the level of Sm-p40, a member of sHSP subfamily, continued to increase from 4.0% in immature eggs to 15.2% in miracidia, whereas the level of HSP70 tended to decrease from immature eggs to miracidia. Moreover, nearly all the HSP identified were seldom identified as excretory/secretory products (ESPs) of mature eggs (Mathieson and Wilson 2010). A precaution shall be taken for explanation of this result because the scarcity of HSP in ESPs may be due to their poor solubility, at least for HSP70 (Weinreb et al. 2001). It was also found that during the transformation of cercariae to schistosomula, of which the surface is accordingly transited from a trilaminate surface to a heptalaminate tegument, HSP70 and other HSP were enriched on the tegument (Sotillo et al. 2015). Using biotin labeling, analysis of surface proteins revealed that HSP was also one category of highly abundant proteins on the tegument of *S. japonicum* adult worm, including HSP10, HSP90, and several HSP60 and HSP70 isoforms (Mulvenna et al. 2010). In response to stresses, flatworm HSP is expressed in different patterns. For instance, after ultraviolet irradiation expression of HSP70 was significantly upregulated, whereas HSP60 and HSP90 were downregulated in cercariae of *S. japonicum* (Yang et al. 2009), suggesting a distinct role in stress responses.

Flatworm HSP shows diversity in structure. Some of them have typical domains or motifs, but some have variants or even lose. Some *Echinococcus* species and *T. solium* possess a large amount of expanded cytosolic HSP70 without an EEVD

motif, which is commonly present in canonical HSP70s and binds to a substrate. Transcriptome data confirmed that these non-canonical HSP70s had almost no expression and seemed to be only expressed under specific circumstances (Tsai et al. 2013). A mitochondrial HSP70 lacking an EEVD motif was also found in *S. japonicum*. Both RT-PCR and Western blot results confirmed that it was expressed at almost all the developmental stages, and particularly highly expressed in the eggs (He et al. 2010). A α–crystallin domain is a determinant of sHSP. A systematic phylogenetic analysis has shown that flatworm sHSP contains two α–crystallin domains, one close to the amino end (N) and the other close to the carboxyl end (C). Moreover, the N or C domains of different species share higher similarity than that of the N and C domains of the same species (Perez-Morales and Espinoza 2015). In trematodes and cestodes, a subclass of sHSP with a truncated C-end extension or HSP70 with a truncated N terminus was also described (Koziol et al. 2009; Liu et al. 2009; Mathieson and Wilson 2010). Although a biological role is largely unknown, it is clear that most of these HSP without typical structures/motifs are a consequence of expanding HSP' functions in response to changed growth environment parasites face during evolution.

In most cases, HSP acts as oligomers or complexes with other proteins and/or chaperones, and posttranslational modifications are an essential factor for association and disassociation of these oligomers or complexes (Carra et al. 2017; Feder and Hofmann 1999), thus affecting their functions. Therefore, organisms have adopted this mechanism to regulate HSP activity during evolution. For precisely playing an appropriate role under certain conditions, HSP needs to be subjected to addition or removal of specific chemical groups post translation, such as phosphorylation, acetylation, ubiquitination, and methylation for HSP70, and phosphorylation, acetylation, methylation, S-nitrosylation, and glycosylation for HSP90 (Cloutier and Coulombe 2013). Analysis of *in vivo* phosphorylation spectra revealed that HSP90 was phosphorylated in schistosomula, and adult males and females of *S. japonicum*, and this modification always occurred at an evolutionarily conserved site (Cheng et al. 2013; Luo et al. 2012). However, the biological implications of phosphorylated HSP90 remain to be elucidated in future.

In eukaryotes, the expression of HSP is tightly supervised by a rather conserved transcription factor known as heat-shock transcription factor 1 (HSF1). Under a thermal stress, HSF1 is transited from a dormant monomer into an active trimer, rendering it to have DNA-binding capacity. Then activated HSF1 interacts with the heat-shock element, a promoter of a *hsp* gene, leading to recruitment of RNA polymerase II, and a cohort of appropriate *hsp* genes are rapidly synthesized until the stress is removed. Upon a stress subsided HSF1 returns back to an inactive state via various modifications (O'Brien and van Oosten-Hawle 2016). Earlier studies showed that *S. mansoni* expressed multiple variants of *hsf1* mRNA possibly by means of alternative splicing, and different isoforms were likely to be differentially expressed at different developmental stages. All the *hsf1* variants had one DNA-binding domain and two leucine zipper motifs, but due to insertions in one of two motifs they might have different DNA-binding activities (Lantner et al. 1998). The same group further demonstrated that schistosome HSF1 seemed to have a specific

recognition pattern, with no interactions with an ideal heat-shock element consensus DNA sequence (nGAAnnTTCnnGAAn). Moreover, the DNA-binding domain itself as well as the elements surrounding it all influenced the specificity of HSF1 (Lardans et al. 2001). Unexpectedly, a recent study showed that HSF1 was mainly localized in the acetabular glands of *S. mansoni* cercariae, an organ involving in invasion, suggesting an additional role beyond acting as a transcriptional factor (Ishida et al. 2014). Apart from a heat-shock element, it has been well established that many other elements/factors, such as an upstream promoter element, a cAMP response element, and IL-6 transcription factors, contribute to regulation of HSP (Prodromou 2016). However, it is poorly understood for HSP in flatworms.

11.1.2 Secretion of Flatworm HSP

With a few exceptions, most HSP seem to lack a canonical secretory signal sequence (Batulan et al. 2016; Cass et al. 2007; Liu et al. 2009; Mambula et al. 2007; Sotillo et al. 2015). Despite the absence of known signal peptides, a growing number of studies have demonstrated that HSP is extensively present in ESPs of a number of flatworms, and some HSP are among the most abundant proteins in secretomes, such as sm-p40 and HSP70 in *S. mansoni* egg secretions (Cass et al. 2007), HSP90α and three HSP70 variants in *S. japonicum* adult worm ESPs (Liu et al. 2009), and sHSP36 in *E. granulosus* adult worm ESPs (Wang et al. 2015).

Secretion of these HSP via non-canonical pathways has been proposed (Cass et al. 2007; Mambula et al. 2007). One of possibilities is to release HSP molecules via extracellular vesicles (EVs). EVs are membrane-bounded vesicles dynamically released by eukaryotic cells, and they encapsulate many active molecules including RNA species, proteins, and lipids. EVs can be internalized by recipient cells, and play a role in intracellular communication within the same species or between pathogens and hosts. Parasitic flatworms can produce EVs via multiple mechanisms, possibly involved in parasitism (Chaiyadet et al. 2015; Cwiklinski et al. 2015; Zheng et al. 2017; Zhu et al. 2016). Consistent with this idea, a few HSP including HSP70 and HSP90 were commonly identified in EVs secreted by helminthes (Buck et al. 2014; Zhu et al. 2016). But this seems to be limited for explanation of a diversity of secreted HSP, possibly due to only the smaller exosome-like EVs or exosomes investigated in these studies, a subgroup of EVs with a diameter of 30–150 nm. Therefore, to further understand a role of EVs in transportation and release of HSP still requires a lot of studies on larger EVs ranged from 100–1000 nm in a diameter secreted by flatworms.

Recently, a lysosomal pathway has been proposed for HSP70 secretion, whereby interleukin 1β and other molecules are released (Mambula et al. 2007). Prior to secretion, HSP70 is first loaded into endolysosomes with the help of ATP-binding cassette transporters. Then HSP70-containing endolysosomes are moved to and fused with the cellular surface, thus leading to cargo release. This model is convincingly supported by the facts that both methylamine and ammonium chloride, inhibi-

tors of lysosomes, remarkably suppressed a secretory rate of HSP70 in cells post heat shock treatment (Mambula and Calderwood 2006). Similarly, HSP27 is likely to be released under the same mechanism, but dephosphorylation is a key step for loading it into lysosomes (Batulan et al. 2016). It is becoming clear that HSP may be secreted under different mechanisms in different cell types or in the same cells under different physiological conditions. There are still lots of gaps in full understanding of HSP release in a lysosome-dependent manner. Whether lysosomes are an active transporter for flatworm HSP is yet largely unknown.

11.1.3 Immunogenic and Immunomodulatory Properties of Flatworm HSP

It is well known that HSP has capacity to stimulate many immune cells, generating pro- and anti-inflammatory cytokines (Kolinski et al. 2016). In flatworms, some HSP, including HSP70 and HSP20, were well recognized by sera of infected animals or humans, suggesting the immunogenic properties (Kouguchi et al. 2010; Mutapi et al. 2011; Wang et al. 2009b; Wang et al. 2015). Systematic analysis of antigen recognition patterns showed that some isoforms of HSP70s strongly reacted with all the isotypes IgA, IgE, IgG1, and IgG4 of humans infected by *Schistosoma haematobium*. However, no reactions with all the isotypes were observed for HSP60, indicating the distinct immunogenicity of different HSP during parasitic infections (Mutapi et al. 2011). *In vitro* experiments manifested that both HSP70 and HSP90 activated mouse bone marrow dendritic cells to upregulate expression of surface markers and cytokines, especially pro-inflammatory cytokines including IL-12p70, IL-6, IL-1β, and TNF-α. Consistently, after stimulation with HSP70 or HSP90, IFN-γ was significantly upregulated in the splenocytes from HSP-immunized mice compared to that from PBS-immunized control (Chung et al. 2017). These results suggest that both HSP tend to induce Th1-biased immune responses. Similarly, a mortalin-like protein from *S. japonicum*, a member of HSP70 subfamily, was also demonstrated to induce the high tilters of specific IgG1, IgG2a and IgG2b isotypes, and the secretion of IFN-γ by the splenocytes from immunized mice (He et al. 2010). However, a contrary expression profile of cytokines was reported in macrophages treated with recombinant HSP70. Compared with LPS, *S. japonicum* HSP70 significantly suppressed expression of L-6, IL-1β, and TNF-α, but remarkably stimulated IL-10 expression (Cao et al. 2016). Due to existence of the different mechanisms of HSP on different immune cells (Bolhassani and Rafati 2008), this discrepancy may result from distinct cell types tested in these studies. There are several members/isoforms of HSP70 subfamily in flukes (Scott and McManus 1999), and they may execute different functions as evidenced by the facts that they are different in expression, tissue distribution, secretion, and induced immune response types. For example, *S. japonicum* mortalin-like HSP70 and *sj648*/HSP70 share 50% identity at the amino acid level, and both are expressed at almost all the

developmental stages. The former could induce both Th1- and Th2-type immune responses, with the higher level of IgG2a than that of IgG1 and IgG2b, but the latter tended to skew towards a Th2-type immune response, with the higher level of IgG1 than that of IgG2a and IgG2b (He et al. 2010; Yang et al. 2012). Therefore, it can't be ruled out that the decreased expression of pro-inflammatory cytokines may be attributed to use of a different member/isoform of HSP70.

The principles of HSP immunogenicity and immunomodulation seem complex and are not yet fully understood. Available data suggest that HSP-client complexes are a key player in induction of protection responses via a cross antigen presentation pathway. Receptor-mediated endocytosis followed by antigen presentation has also been proposed for Gp96, a member of HSP90 subfamily (Bolhassani and Rafati 2008). Another explanation is that HSP acts as molecular mimicry, which is well recognized by immune system. It is supported by the facts that HSP is involved in a number of autoimmune disorders (Chebotareva et al. 2017). *S. japonicum* HSP60 sharing a common T cell epitope with humans also exemplifies this hypothesis (Wang et al. 2009a). Additionally, activation of immune cells is also depend on HSP' capacity of upregulating costimulatory surface markers and cytokines, and on HSP' association with Toll-like receptors (TLRs) (Bolhassani and Rafati 2008).

11.1.4 Roles of HSP in Flatworms and Their Infections

An increasing body of studies has suggested the multi-functions of HSP in the development of flatworms and during their infections. Sm-p40 is likely to offer protection for *S. mansoni* eggs and miracidia from extreme growth conditions, whereas HSP70 acts as a stabilizer to aid protein maturation and maintain macromolecules' structure (Cass et al. 2007; Mathieson and Wilson 2010; Sotillo et al. 2015). Moreover, HSP70 is also supposed to protect *S. japonicum* cercariae from ultraviolet irradiation damage (Yang et al. 2009). HSP90, at least partially, is present in a phosphorylated form in *Schistosoma* species (Cheng et al. 2013; Luo et al. 2012). *In vitro* silencing of HSP90 led to remarkable downregulation of a number of signal molecules that were predicted to interact with it, such as 14–3-3 family protein and cell division cycle 37 homologue. Furthermore, both *in vitro* and *in vivo* treatments with celastrol, an inhibitor for HSP90, decreased the viability of parasites, with worm burden reduction and liver egg reduction by 57% and 67%, respectively (Cheng et al. 2013). Taken together these results suggest a key role of HSP90 in schistosome development.

Tegument is a complex structure coating onto the surface of flatworms, and plays a role in many processes, including nutrient uptake, immune response modulation and evasion. Fluke tegument contains a plethora of proteins and lipids, some of which are unique and absent in hosts (Van Hellemond et al. 2006). Of them, HSP is one group of enriched tegumental proteins (Mulvenna et al. 2010; Sotillo et al. 2015). Recently, two different isoforms of tegumental HSP70 were shown to bind to human low-density lipoprotein (LDL), but their role remains elusive (Pereira

et al. 2015). The binding of LDL to HSP70 may be explained by forming a barrier that protects parasites from immune attacks (Van Hellemond et al. 2006). Another possibility is that the binding may be associated with transportation of apoprotein B, which is present in LDL and interacts with HSP70 (Pereira et al. 2015). As flukes are not able to generate *de novo* long-chain fatty acids and sterols, of high interest is to determine whether or not the HSP70-LDL interactions are involved in trafficking lipids in future studies.

A role of flatworm HSP in immunity is well studied, and HSP is involved in immune responses by many different mechanisms. To avoid clearance by a host, one of strategies that parasitic flatworms have taken is to downregulate host immune reactions. During chronic *Schistosoma* infection, immunosuppression occurs and a Th2 response profile is dominant (Mitchell et al. 2008). *S. japonicum* HSP60 was found to be an active player in this Th2-dominant immunosuppressive response by promoting proliferation of $CD4^+CD25^+Foxp3^+$ T cells (Wang et al. 2009a), a subpopulation of T cells that are essential for adjustment of peripheral tolerance and exacerbated immune responses. *S. japonicum* HSP60 has a 24-amino acid fragment (SJMHE1) identical to human and mouse HSP60s, which comprises overlapping T cell epitopes. Both *in vivo* and *in vitro* studies demonstrated the capacity of this short peptide in induction of differentiation from $CD4^+CD25^-$ T cells to $CD4^+CD25^+Foxp3^+$ T cells, and in enhancement of immunosuppressive activities of $CD4^+CD25^+Foxp3^+$ T cells. Moreover, knockout of TLR2 rather TLR4 gave no increase of $CD4^+CD25^+Foxp3^+$ T cell proportions in mice immunized with SJMHE1, suggesting a critical role of TLR2 in specific promotion of Treg cell development (Wang et al. 2009a). This increased immunosuppression of Treg may be accomplished by direct binding of HSP60 to TLR2 on the cellular surface (Kolinski et al. 2016). In agreement with these findings, the HSP60-derived peptide SJMHE1 could suppress delayed-type hypersensitivity in mice induced by ovalbumin. Further investigation revealed that the suppression was mediated by transition of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ T cells in the periphery, which secreted the high levels of anti-inflammatory cytokines IL-10 and TGF-β1. These cytokines elicited inhibition of responder T cell proliferation in mice with delayed-type hypersensitivity (Wang et al. 2016b).

11.1.5 HSP as Vaccine and Diagnostic Targets for Parasitic Diseases by Flatworms

Due to immunogenic properties, HSP is considered to be promising candidates for vaccines and diagnostic reagents (Table 11.2). HSP20 has a diagnostic potential as a biomarker for human cystic echinococcosis. A recent study showed that *E. granulosus* HSP20 reacted well with 64% (61/94) of positive sera, but not with 37 sera from healthy humans. Despite low reactivity with positive sera, the level of specific anti-HSP20 IgG in sera is dynamically correlated to the state of the disease. At the

Table 11.2 Parasitic flatworm HSP as promising drug, vaccine and diagnostic candidates

Species	Disease	HSP	Efficacy	Target	Reference
Echinococcus granulosus	cystic echinococcosis	HSP20	Diagnosis, low positive rate (64%, 61/95) but linear correlation to the state of the disease	human sera	Vacirca et al. (2011)
Schistosoma japonicum	schistosomiasis	mortalin-like HSP70	DNA vaccine, 25.9% worm reduction, 43.49% liver egg reduction, 44.8% intestinal egg reduction	mouse	He et al. (2010)
		SjHSP70	Recombinant protein vaccine, >25% worm burden reduction	mouse	Duan et al. (2015)
		HSP90	Drug target, 57% worm burden reduction, 67% liver egg reduction	mouse	Cheng et al. (2013)

end of follow-up, no HSP20-specific antibodies were detected in echinococcosis patients with surgery and/or pharmaceutical treatments (Vacirca et al. 2011). HSP70 is a promising vaccine candidate. In mice, immunization of a DNA vaccine harboring mortalin-like HSP70 elicited Th1-dominated immune responses, and *S. japonicum* worm burden, liver and intestinal egg loads reduced by 25.9%, 43.49% and 44.8%, respectively (He et al. 2010). Similarly, immunization of recombinant HSP70 antigen alone also induced a protective Th2-biased response in BALB/c and C57BL/6 mice challenged with *S. japonicum* cercariae, with worm burden reduction by >35% and >25%, respectively (Duan et al. 2015). It should be mentioned that these challenge trials were conducted only in mice, so the protective efficacy induced by these HSP would need to be further assessed in natural hosts, including sheep and/or cattle.

11.2 Conclusions

The presence of extracellular HSP is expanding our knowledge on their biological functions beyond molecular chaperones. It is becoming clear that HSP has close links to occurrence and development of many diseases including parasitic diseases, favoring them to be pharmaceutically targeted for treatment. Using small molecular inhibitors, intervention can rely on either acting on the N- or C-terminal binding domain of HSP, or regulating HSP-co-chaperone interactions or HSP-client interactions (Shrestha et al. 2016). At present, only a few studies on flatworm HSP as a drug target have been reported so far (Cheng et al. 2013), and development of effective HSP-targeting antagonists is of high veterinary interest. One of key questions is how to effectively deliver drugs to targets. Utilization of nanocarries, such as liposomes, polymers, and lipid micelles, to encapsulate active antagonists is a

prospective way. For example, a plenty of nanomedicines targeting HSP90 have been developed, and some are already at the preclinical stage (Sauvage et al. 2017). It is also possible to use these antagonists to pinpoint a role of flatworm HSP during parasitism, which in turn aids us to better control parasite transmission.

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Chapter 12
Heat Shock Proteins: Role, Functions and Structure in Parasitic Helminths

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Abstract Most helminths are parasitic in humans and domestic animals and are responsible for considerable economic losses and public health problems worldwide. In parasites, unlike most organisms, heat shock proteins (HSP) are involved in the physiological phenomenon and passage of helminths between host and environment that is part of their life cycle. The sequence of these proteins being highly conserved across a wide variety of organisms is related to their potential role as immunogenic intracellular molecules. In this chapter, we describe the functional properties of HSP and their roles in parasitic helminths. In addition, we summarize the stress response, development, comparison of the sequences and structure of HSP in some parasites, and phylogenetic markers.

Keywords Development · Heat shock protein · Parasitic helminths · Phylogenetic marker · Structure · Temperature

Abbreviations

ATP	Adenosine triphosphate
GA	Geldanamycin
HSC70	Constitutive heat shock protein 70
L3	Third-stage larvae
L4	Fourth-stage larvae
SjGST	Glutathione S-transferase

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12.1 Introduction

Protozoan and helminths are two major taxa acting as parasites that cause various diseases in humans. In the Global Burden of Disease Study 2013, the tropical disease malaria was the major parasitic-protozoan disease killer, causing over 850,000 deaths, particularly due to the protozoan parasite *Plasmodium falciparum*, followed by Leishmaniasis, Cryptosporidiosis, Chagas disease, African Trypanosomiasis, Schistosomiasis and Ascariasis. About 50,000 species of helminths have been described in a wide range of host species. Helminths are commonly known as parasitic worms, Nemathelminthes (nematodes), and include flatworms (platyhelminthes), namely trematodes (flukes) and cestodes (tapeworms). The most abundant parasitic species are nematodes; serious infections include Ascariasis, filiariasis, and hookworm diseases. Schistosoma, a genus of trematodes, commonly known as blood flukes and causative agents of schistosomiasis (intestinal or urinary depending on the species), have infected more than 200 million people around the globe (WHO 2015). Schistosomiasis and other helminthiases may contribute to the transmission of malaria co-infections (Mbah et al. 2014). Parasitic infections result in morbidity, mortality and economic losses (reduced meat, milk, and agricultural production), and treatment is challenging because of drug resistance, poor efficacy, toxicity and high cost.

Most parasitic helminths have a complex life cycle that involve differentiation through several stages of development and transmission through two or more hosts; therefore, their elimination is difficult. In most cases, third-stage larvae (L3) of parasitic nematodes are the infective stage, when the larvae are transferred from cold-blooded animals (fish, snails and insects) to homeothermic animals such as *Angiostrongylus cantonensis* and *Wuchereria bancrofti* (Boakye et al. 2004; Kim et al. 2014). Some soil-transmitted nematodes must live in a harsh outside environment until they find and successfully infect an appropriate host such as *Strongyloides stercoralis* (Knopp et al. 2008). After successful invasion, parasites are exposed to adverse temperature, pH and osmotic pressure between host and environmental factors but also the host immune system, which might cause enormous stress. Parasites have to switch several molecular and physiological features to respond to changes in the surrounding environment (Perry and Moens 2011). These extreme environmental changes lead to a swift and specific physiological transformation that results in changes in surface membranes and glycosylation shedding from cercaria to schistosomulum (Řimnáčová et al. 2017). These changes are accompanied by transcriptional changes (Gobert et al. 2010).

HSP are a group of highly conserved proteins found in almost all living organisms from bacteria to eukaryotes. They are cellular proteins that are present under normal conditions, and their expression increases under stress (Asea et al. 2000; Devaney 2006). HSP have been grouped according to their molecular weight into several families: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small HSP. Recently, inhibitors of Hsp90 and Hsp70 were developed as drug targets for the treatment of cancer and parasitic diseases (Kaur et al. 2010; Edkins and Blatch 2012). HSP are also implicated in the clinical characteristics of metabolic syndrome components such as

diabetes, obesity and stroke (Kim and Yenari 2013). HSP are ubiquitous in all species and are highly conserved; they provide a useful model for evolutionary studies (Gupta 1995).

This chapter reviews the roles and functional properties of HSP in parasites and their expression profiles under different stress conditions such as different stages of the parasite life cycle and under cold and heat shock. We emphasize the role of two well-characterized proteins, Hsp70 and Hsp90. In addition, we present an analysis comparing the sequences and crystal structures of HSP in parasites by using as a model L3 larvae of *Anisakis pegreffii*, a dominant species found in various marine fish and causing human anisakiasis.

12.1.1 General Characteristics of HSP

HSP are an essential molecular chaperone in eukaryotic organisms and are abundant in unstressed cells, constituting 1% to 2% of the cytosolic proteins (Pratt 1998). They are important in many cellular processes including protein folding, signal transduction, apoptosis, growth and development. Their main function is to help denatured proteins refold or to target them for degradation and in some cases, their multimerization into oligomeric structures (Narberhaus 2002). They play key roles in response to many stresses such as thermal stress, heavy metal exposure and parasite infections and protect organisms against environmentally induced cellular damage (Devaney 2006; Grabner et al. 2014).

Although HSP are a highly conserved family of proteins, the function of Hsp90 seems to vary among different parasites. Some nematodes may possess an atypical Hsp90 that, as demonstrated by treatment with the inhibitor geldanamycin (GA), directly binds to the ATP-binding pocket in the N-terminal domain of Hsp90. GA is a naturally occurring ansamycin compound produced by *Streptomyces hygroscopicus* (BeBoer and Dietz 1976) and was used to probe the function of the Hsp90 chaperone in many studies (Whitesell et al. 1994; Workman 2003). Obligate parasites or those living in the environment enclosed within an egg could bind GA but not free-living nematodes and parasites with free-living larvae (Him et al. 2009). The results of their study suggested that the ability of Hsp90 to bind GA was associated with the life cycles of different nematodes. Hsp90 is a multifaceted role within the cell, and the rapid evolution of the Hsp90 gene relates to other key cellular functions (Him et al. 2009).

Hsp70 proteins are induced in response to stress, called inducible Hsp70, although some Hsp70 species are constitutively expressed in cells, called cognate Hsc70 (Lindquist and Craig 1988). Hsp70 plays an important role in the host–parasite interaction. It is as an inducible protective protein and an antigen in parasites (Maresca and Kobayashi 1994). Among these proteins, Hsp70 is an immunodominant antigen during infections caused by different pathogens (parasite and virus) (Bolhassani and Rafati 2008). It can also target vaccine antigens to antigen-presenting cells to improve vaccine immunogenicity (Wan et al. 2004).

12.1.2 Expression of HSP under Stress Conditions

The most important factor affecting the abundance and distribution of nematode species around the world is temperature (Basáñez et al. 2012). In particular, nematodes have low tolerance to heat. Hsp90 was expressed in *A. pegreffii* at 50 °C (Chen et al. 2014). In heat stress experiments, *Anisakis* larvae changed their appearance from bright translucent to opaque white, the bodies were ruptured and the gut broke through the mouth opening. Increased HSP expression during short-term heat stress may be due to the heat stress stimulating and quickly initiating the transcription or translation of the HSP to protect cells against heat stress (Dangi et al. 2014). The transcriptional expression of *A. pegreffii* Hsp90 showed a clear time-dependent response under heat stress: the expression significantly increased after 1 hr. at 4 °C (Chen et al. 2014). *A. pegreffii* Hsp90 is predominantly expressed after heat stress as compared to cold stress. Hence, Hsp90 might be responsible for protecting the host with the different intensity of heat and cold stresses. In *Ostertagia ostertagi*, the OoHsp18 gene was significantly upregulated in response to heat shock, with no effect on OoHsp18 levels during other types of stress such as exposure to 2% H_2O_2 or 150 μg/ml of the antihelminthic compound levamisole (Vercauteren et al. 2006).

12.1.3 Expression of HSP during Parasite Development

Almost all living organisms do not undergo heat shock during developmental, but for parasites that are transmitted between cold-blooded animals (mosquitoes, insects or fish) to homoiothermal animals, this is a frequent physiological occurrence. Besides increased synthesis of HSP in response to an increase in temperature, HSP of parasites also play important roles in differentiation. Hsp70 is one of the most important HSP whose expression is rapidly induced by heat exposure or other stress (Lindquist and Craig 1988). In *Schistosoma mansoni*, the expression of Hsp70 can be fleetingly induced by stress and temperature (a shift to 42 °C) during the cercariae–schistosomula transformation and in adult worms (Neumann et al. 1993).

As well, HSP expression is inducible during several stages of development in some parasites, which suggests that they play unique biological roles in certain stages of the life cycle. In *A. pegreffii*, Hsp70 and Hsp90 mRNA and protein levels were higher in L4 than L3 larvae (Chen et al. 2015). The expression of ApHsp70 was higher than ApHsp90 in L4 larvae, so these differences may be due to the different regulatory systems between Hsp70 and Hsp90 in different development stages. ApHsp70 and ApHsp90 are important proteins acting against heat stress, and increased transcriptional activity in response to L4 larvae might be important for the development of *A. pegreffii* (Chen et al. 2015). In one experiment, *Brugia malayi* was introduced into the skin of a human host via mosquitoes, and L3 filarial larvae developed into adults; the protein expression of BmHsp70 was higher in microfilariae

and adults than L3 larvae (Bennuru et al. 2011). In *Meloidogyne artiellia*, Hsp90 is constitutively expressed in all stages but at higher levels in eggs and L4 larvae (De Luca et al. 2009). The transcript levels of NbHsp70 and NbHsp90 of the parasitic nematode *Nippostrongylus brasiliensis* were detected throughout the nematode's lifecycle and found closely associated with development (Arizono et al. 2011). The Hsp70 of *Schistosoma japonicum* is widely expressed in all development stages (Yang et al. 2012). In *Trichinella spiralis*, Tsdaf21/Hsp90 protein is abundantly and consistently expressed in all stages (Yang et al. 2013). The spontaneous expression of HSP is an essential part of the host development and the stress response. Recently, inhibitors of Hsp90 and Hsp70 were a focus as drug targets for the control of parasitic diseases (Edkins and Blatch 2012).

12.1.4 Structure Analysis

The function of a protein depends on a 3D structure. The structure domains of Hsp70 and Hsp90 are highly conserved across species. Hsp70 contains three domains: an N-terminal domain and a middle domain, which consists of adenosine triphosphate (ATP) and a substrate binding site, and a C-terminal domain that provides a lid for the substrate domain. The substrate binding site and ATPase domain of Hsp70 are largely conserved in many species, with a variable C-terminal region (Desai et al. 2010). In *A. pegreffii*, ApHsp70 has a Y-shaped structure (Fig. 12.1a) and could be superimposed on the template structure of bovine Hsp70 (PDB id:1YUW). ApHsp70 has 28 beta sheets and 13 helices in the bovine homology model (Chen et al. 2015). In a similar way, Hsp70 homologous have about 28 sheets and 13 helices within other organisms. (Desai et al. 2010). Based on the sequence similarity and structural modeling, Hsp70 protein in evolution is highly conserved for maintaining structural conformation.

Hsp90 is a homodimer, and each monomer contains three flexibly linked regions, namely an N-terminal domain (25 kDa), a middle domain (35 kDa) and a C-terminal dimerization domain (12 kDa) (Nemoto et al. 1997). The charged linker located between the N-terminal and middle domain in eukaryotic Hsp90 varies in amino acid composition and length depending on the species. The C-terminal end contains the MEEVD motif, which provides the binding site for interaction with co-chaperone molecules. ApHsp90 has a dimer structure (Fig. 12.1b) and could be superimposed on the template of yeast Hsp90 (Chen et al. 2015). Structural modeling of ApHsp90 showed a long loop from residues 216 to 291. Computer analysis of the deduced protein sequence revealed a variable sequence of the loop region as compared with ApHsp90 from other species (Fig. 12.1c). From residues 229 to 248, EKKEEEQKKEGEVEDVGEDE is a genetic marker and an epitope, with sequence identity between ApHsp90 and the Hsp90 for other nematode species ranging from 70% to 76% and 55% for yeast and humans. The 20 amino acid sequence may be useful as an antibody for detection of *Anisakis* nematodes.

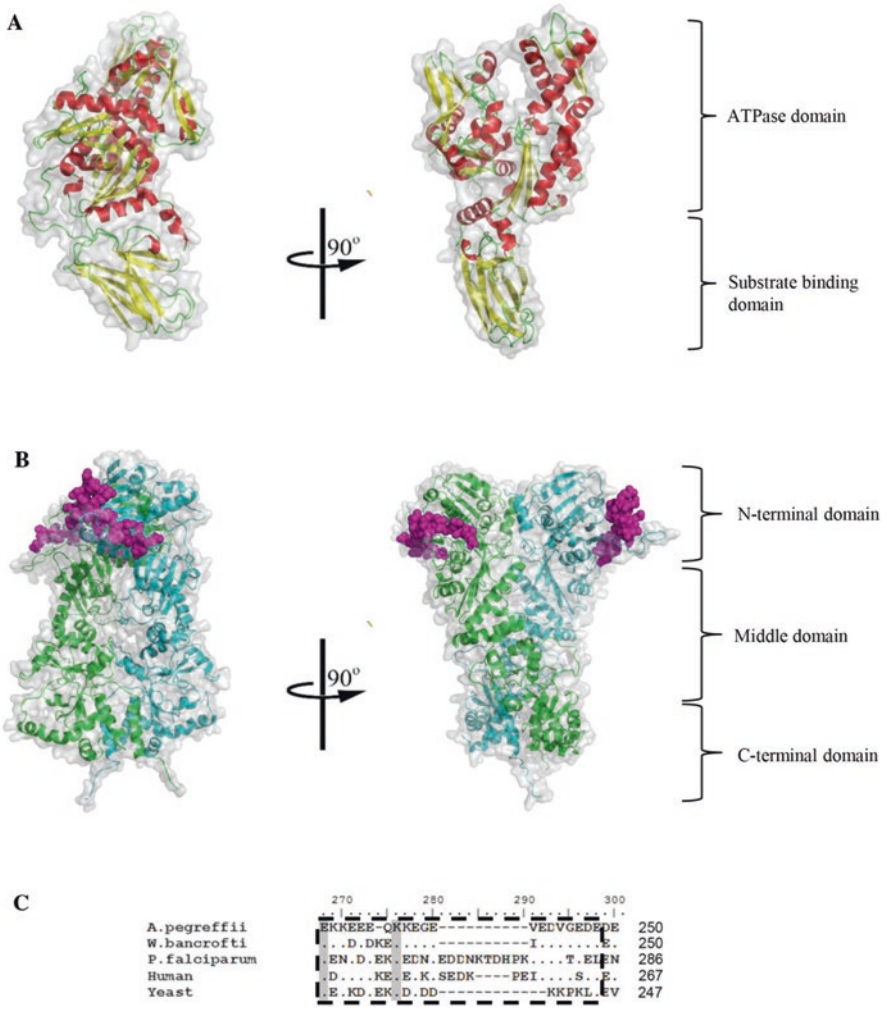

Fig. 12.1 Molecular surface and ribbon diagrams of the modelled structures of *Anisakis pegreffii* heat shock protein. (**a**) The structure of Hsp70 is modelled with the template structure of bovine Hsp70 (PDB id: 1YUW) and shows a Y-shape. The α-helices are depicted in red and the β-sheets in yellow. (**b**) The structure of Hsp90 is modelled based on the template structure of yeast Hsp90 (PDB id: 2CG9) and represents a dimer. The long loop from residues 229–248 is indicated by magenta sphere. The molecular surface and diagrams were generated using PyMOL. (**c**) Multiple sequence alignment shows high variation of Hsp90 proteins in residues 229–248 of *A. pegreffii* with other four different organisms. The high variation region is showed in a dashed box. *A. pegreffii- Anisakis pegreffii* (GeneBank accession number: KF840393); *W. bancrofti- Wuchereria bancrofti* (EJW88125); *P. falciparum- Plasmodium falciparum* (AAA66178); Human- *Homo sapiens* (BAF83804); Yeast- *Saccharomyces cerevisiae* (P02829)

12.1.5 Hsp70 and Hsp90 Genes Are Useful as Phylogenetic Markers

Both Hsp70 and Hsp90 are considered useful in phylogenetic analysis because they are highly conserved throughout prokaryotes and eukaryotes. Hsp70 is widely used as a phylogenetic marker because of the highly conserved sequences and the major molecular chaperone function, in conjunction with an ancient conserved function, so it is a useful system for investigating deep evolutionary relationships (Gupta and Golding 1993). Hsp70 has been used with all monophyletic groups of metazoan, and sequences available for some species were consistently recovered. Hsp70 has also been used for phylogenetic analysis of different protozoan parasites (Fraga et al. 2010). However, Hsp70 was used as a phylogenetic marker with caution, because the paralogy distorts phylogenetic relationships (Krenek et al. 2013). Hsp90 is a proper phylogenetic marker analyzed in a variety of organisms and has a good balance of sequence conservation and diversity. It has distinct domain and signature sequences that allow for differentiation between Hsp90 and other family members. Some potential problems with use of paralogous genes such as Hsp70 are not an issue with Hsp90 (Skantar and Carta 2004). Hsp90 sequences from several nematodes have been phylogenetically evaluated (Him et al. 2009; Chen et al. 2015).

12.1.6 HSP Inhibitors and Parasitic Diseases

HSP are expressed at abnormal levels in several pathologic conditions, such as obesity, type 2 diabetes, neurodegenerative disease, autoimmune diseases and cancer. As major antigens of parasites, once recognized by the host immune system, they can become targets for host B and T cells (Maresca and Kobayashi 1994). HSP have been detected during the course of many parasitic infections (e.g., *Schistosoma*, *Echinococcus*, *Strongyloides*, *Trichinella*, *Onchocerca*, filarial nematodes). For example, in the trematode *S. japonicum*, a glutathione S-transferase (SjGST) DNA vaccine combined with mortalin-like protein/Hsp70 (SjMLP/Hsp70) conferred 30% and 60% reduction in worm and egg burden in immunized mice (He et al. 2010). The Hsp20 of *Echinococcus multilocularis* was recognized by 100% of sera samples from dog, which suggests its efficacy as a vaccine candidate (Kouguchi et al. 2010). The unique species-specific motif of Hsp90 might provide novel insights for identifying selective drug target sites (Faya et al. 2015). The Hsp90-specific inhibitor GA was reported to kill adult worms and microfilariae of *Brugia pahagi* (Taldone et al. 2009). In liver fluke *Clonorchis sinensis*, rCsHSP70 and rCsHSP90 could be considered vaccine candidates because they activated the host immune response such as the T helper 1 cell response via cellular uptake and induction of antigen presentation (Chung et al. 2017).

12.2 Conclusions

In parasites, HSP play important roles in the maintenance of motility and proliferation, stage-specific gene expression, host-cell invasion and stress response. The effect of disruption of Hsp90 on client protein degradation and cell death has been well investigated. Knowledge of the structure and functional pathway of Hsp90 would greatly aid in the effective design of drugs to inhibit parasitic Hsp90 activity. However, Hsp70 and Hsp90 remain useful markers for phylogenetic analysis in parasitic helminths.

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Chapter 13
Heat Shock Proteins and Blood-Feeding in Arthropods

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Abstract The blood of endothermic vertebrates constitutes the main, or even the only food for many arthropod species. Even though blood is a food rich in nutrients and in most cases sterile, its consumption is associated to many stressing factors. Energetic, thermal, osmotic and oxidative stresses are among the consequences for arthropods of the rapid ingestion of large amounts of warm blood. To cope with these stressors, these animals have developed different physiological and molecular mechanisms allowing the reduction of the stress or the reparation of the infringed damage. Among the first, specific mechanisms of thermoregulation and rapid excretion have been identified. The rapid synthesis of HSP following each feeding event make parts of the mechanisms of molecular reparation. The increase in the HSP70 levels varies across species from about 3 to around 17 times the basal level. This variability in the molecular response is plausibly associated to the occurrence or not of complementary mechanisms for reducing the effect of the stressor, as for instance, thermoregulation. The reduction of HSP70 or HSP70/HSC70 expression does not affect the blood meal size, but impairs blood digestion by the insect.

Keywords Disease vectors · Haematophagy · HSP · Kissing-bugs · Mosquitoes · Thermal stress · Thermoregulation

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Abbreviations

AM	Anterior midgut
dsRNA	Double-stranded
RNA	HCP70
HSCP	Heat-shock cognate protein 70
HSP	Heat shock proteins
HSP70	Heat shock protein 70
IMD	Immune deficiency
mRNA	Messenger RNA
PM	Posterior midgut
RNAi	RNA interference

13.1 Introduction

The blood of vertebrate animals represents a highly nutritive element where proteins account for more than 90% of total dry weight and, except for the eventual presence of parasites, otherwise sterile. This made many arthropods to adopt it as a main (e.g., mosquitoes) or even only food along their whole life (e.g., kissing-bugs). Nevertheless, blood is not freely available in nature, but it circulates inside vessels hidden under the skin of active animals, capable of defending themselves, and normally larger than the blood-sucking organism. To feed on vertebrate blood is a risky task, which requires being able of solving several major problems related with obtaining food from hosts, which can play, at the same time, the role of predators. As a consequence, strong selective pressures have modelled specific morphological, physiological and behavioural adaptations in animals having a haematophagous way of life.

13.1.1 Blood-Feeding as a Stressful Event

Most blood-sucking arthropods take big amounts of blood, in relatively short times, in order to minimize the risks associated to frequent host search and to reduce the duration of contact with hosts. This rapid ingestion of a mass of fluid, which can account for many times their own body weight also implies a significant stress for the animal. This stress has multiple forms and physiological targets. First, the pumping of a large amounts of blood to the intestine in a short time generates an important mechanical stress associated a sudden increase in metabolic rate (Leis et al. 2016). Second, the intake of blood from endothermic vertebrates implies the transfer of a big amount of heat and, as a consequence, thermal stress (Benoit et al. 2011; Lahondère and Lazzari 2012, 2013; Lahondère et al. 2017). Third, the water

and ion balance due to the contrast between the excess water input with the blood intake and the dehydration during the off-host periods (Maddrell 1991; Benoit et al. 2010). Fourth, big amounts of blood imply high amounts of toxic or potentially noxious elements entering into the body (Sterkel et al. 2017). Finally, as haemoglobin is a major blood protein, representing about 60% of the proteins in the blood, its digestion produces big amounts of heme, which is a pro-oxidant molecule (Oliveira et al. 2011).

In addition, vertebrate host blood is the route used by several species of parasites to infect hematophagous arthropods, which development will trigger a variety of cellular stresses causing an immune response in these invertebrates.

All these stressors induce specific adaptive responses reducing their impact and also act as selective pressures for the development and support of particular physiological strategies for overcoming them. Among these responses, the synthesis of HSP triggered by feeding plays a major role in protecting the cellular integrity and reducing the physiological impact of stress associated to the ingestion of a blood meal.

13.1.2 Thermal Stress, Thermoregulation and Molecular Reparative Measures

The first evidence of the occurrence of thermal stress during feeding in haematophagous insects were obtained relatively recently (Benoit et al. 2011). The variation of the temperature of the body during the procurement of a blood meal was measured in different species of blood-sucking insects, including two mosquitoes, *Aedes aegypti* and *Anopheles gambiae* using thermocouples. A steady increase of the body temperature, reaching peak values of up to 10 °C above the initial temperature of the insect body, was verified a few minutes after the beginning of blood ingestion. After gorging, the body temperature decreases gradually to come back similar to that of the environmental. Depending on the values of environmental temperature, which is the initial temperature of the insect, and that of the blood, the amplitude and dynamics of heating and cooling may vary due to thermodynamic reasons, as the heat conductivity of the body wall or the presence or absence of wind.

In some species, however, specific mechanisms for dissipating the excess of heat exist. For instance, in the malaria vector *Anopheles stephensi*, a thermoregulatory mechanism allows dissipating a part of the heat entering into the mosquito body with the ingested blood, reducing the potential thermal stress to which internal organs may be exposed. During feeding, this mosquito emits droplets of a fluid composed of urine and blood, which are retained at the end of the abdomen. The evaporation of the fluid dissipates the excess of heat, reducing the temperature of the whole body; cooling in particular the abdomen of the mosquito (Lahondère and Lazzari 2012, 2013).

Other physiological responses of insects to overheating during feeding include molecular changes, as a rapid increase in the level of HSP (Benoit et al. 2011). As in many other organisms, mosquito Hsp70s have been shown to increase during environmental stress (Gross et al. 2009; Benoit et al. 2010). Benoit and co-workers (2011) showed that, correlated with feeding and the associated elevation of the body temperature, a synthesis of heat-shock proteins occurs in *Aedes aegypti* in the few hours following a blood meal, in particular of Hsp70. In this species, the Hsp70 synthesis peaks 1 h after feeding, reaching its maximal expression in the mosquito midgut, where the relative amount of Hsp70 increases about 7 times after feeding. Similar increases in Hsp70 were showed immediately after blood feeding in *Culex pipiens* and in *Anopheles gambiae*, as well as in the bed bug *Cimex lectularius*. Nevertheless, this increase, measured as the relative increase of mRNA by Northern blot, is not identical in different species. Whereas in *Aedes aegypti* and *Culex pipiens* the relative level increases between nine and ten times, in *Anopheles gambiae* only three times. The vector of leishmaniosis *Lutzomia longipalpis* increases by a factor of 17 the level of HSP70 after the haematophagous act (Aguiar-Martins 2015), whereas in the kissing bug *Rhodnius prolixus* increases about 3.5-fold in the insect midgut (Paim et al. 2016). This important difference in the synthesis of HSP across species, could be related to the adaptation to different environments of different species, but also to the strategy to cope with thermal stress, i.e., thermoregulation or molecular reparative measures.

13.1.3 HSP70 (HSP70/HSC70) and Blood Feeding

One of the common physiological responses of various organisms to stress situations is the rapid increase in the expression of Heat Shock proteins (HSP) transcripts. Among the various types of Heat Shock proteins, HSP70 has been the most well characterized and studied in several insect species. As mentioned above, the hematophagous insects show an increase of 3-17fold in HSP70 expression after the blood meal.

In *R. prolixus* knocked down for HSP70/HSC70 maintained in starvation after HSP70 knockdown prematurely died in a short interval of 8 days (within 32–40 days after HSP70 dsRNA injection), whereas control insects gradually died over an interval of 44 days, from 42nd to the 86th day after injection (Paim et al. 2016). The authors suggested that these insects may be dying due to their inability to counteract the stress caused by prolonged starvation. Kollien and Billingsley (2002) had reported the up-regulation of HSP70 expression in *Triatoma infestans* under long-lasting starvation. Triatomine bugs ingest water only during the blood feeding so the hydric stress induced by long periods of starvation is very critical for these insects (Rolandi et al. 2014). HSP70 RNAi knockdown in *Aedes aegypti* significantly reduced the dehydration tolerance in these haematophagous insects (Benoit et al. 2010).

Furthermore, it was observed that *R. prolixus* fed after HSP70 knockdown died even earlier, within 21–27 days post dsRNA injection. Blood feeding induced 100% mortality in engorged HSP70 knockdown insects, whereas all controls were able to normally moult to the adult phase in the same period. A mortality rate of 100% was also observed in engorged HSP70-knockdown adult females (which need to feed but do not perform ecdysis).

Physiologically normal *R. prolixus* nymphs, during their feeding, may ingest up to 9 times their initial host blood weight. This blood passes through the anterior intestine and reaches the first portion of the midgut: the anterior midgut (AM), where it is stored (Orchard 2006). In its engorged state, the insect has limited mobility and becomes more vulnerable. Thus, the insect need to quickly eliminate water and salts excess to reduce its body volume (Maddrell and Gardiner 1976). In this context, the AM acts to promote rapid diuresis of the water and ions present in the blood through the AM epithelium to the haemolymph and subsequently, from the haemolymph to the Malpighi tubules. Urine flows to the distal portion of the Malpighi tubules, where K^+ is reabsorbed, and continues to the rectum, where it is eliminated at regular intervals. The integration of the events associated with diet and diuresis is coordinated by a combination of hormones, with serotonin being one of the main factors of this coordination, being present in the nervous system, salivary glands and AM of *R. prolixus* (Brugge et al. 2008). Among other functions, serotonin stimulates the contraction of the digestive tract muscles, induces the cuticle plasticization, allows the insect to accommodate a larger volume of ingested blood and stimulates the transport of water and ions from the AM to the haemolymph (Orchard 2006). In addition to regulating the volume of water and ions present in the blood repellent, AM is still involved in carbohydrate digestion, haemolysis, and storage of glycogen and lipids (Billingsley 1988). Consequently, the blood nutrients are concentrated in the AM and pass in small portions to the posterior midgut (PM), where digestive proteases are secreted and also where nutrient uptake and storage occurs (Billingsley 1988). The digestion process in PM is probably mediated by the endocrine system, since Okasha (1964, 1970) demonstrated that decapitated insects had low levels of proteases in PM. In *R. prolixus* adults, the peak of protease activity occurs on the 4th day after feeding and then gradually decreases. In adult females, this peak of protease activity in PM refers to the vitellogenesis cycle in the ovaries, where several parameters such as protein transfer in the oocytes and trophocytes growth reach a peak on the 4th day after feeding. Although the regulation of these two events is independent, this apparent relationship between them is quite appropriate, since high protein activity in the PM leads to increased amino acid availability in the haemolymph for protein production during vitellogenesis (Persaud and Davey 1971).

In mosquitoes, it is known that there are pathways of digestion regulation in the midgut involving the interaction of insulin and TOR ("target of rapamycin"). Insulin-like peptides are released from the brain of mosquitoes after blood ingestion, interact with TOR and induce the expression of late trypsin-like enzymes in the midgut, which are the enzymes responsible for most of the digestion in the mosquitoes midgut (Gulia-Nuss et al. 2011). In the transcriptome of *R. prolixus* digestive

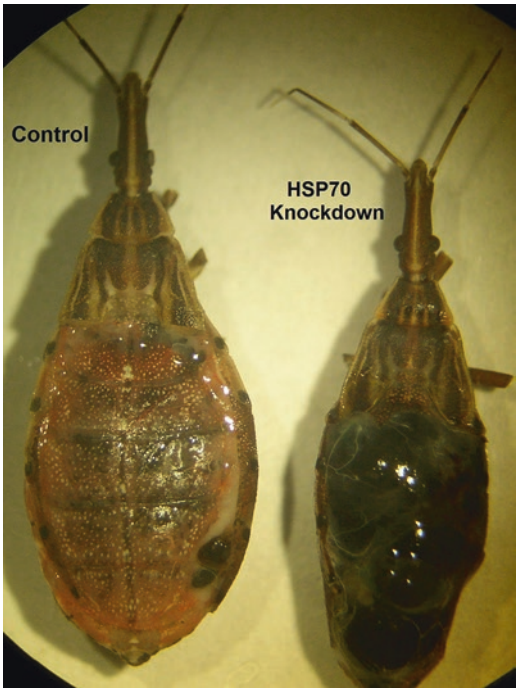

Fig. 13.1 Internal aspect of *R. prolixus* 5th instar nymphs dissected at the 14th day after blood feeding (the dorsal exoskeleton was removed in each bug), showing a new pink cuticle forming under the older cuticle in the control nymph (*left bug*) and the midgut still full of blood, occupying most of the abdominal cavity of the HSP70 knockdown insect (*right bug*), indicating that the ingested blood was not adequately processed and digested when HSP70 was suppressed in this insect

tract, TOR transcripts coding for a regulatory protein kinase of several cellular processes were found, such as cell growth, proliferation and survival (Ribeiro et al. 2014). The presence of TOR transcripts in *R. prolixus* gut suggests a possible role of the TOR signalling pathway in the regulation of protein digestion in the PM of this triatomine. However, the functioning of the mechanism of digestion signalling in *R. prolixus* is still scarce understood.

Several parameters related to the ingestion, processing and blood digestion were investigated to understand the mechanisms by which the blood ingestion caused the premature death of HSP70-supressed insects. The weight loss in the first hours after blood intake (which corresponds to the diuresis peak) was not affected by HSP70 suppression, but the blood processing and digestion were significantly impaired. At the 14th day after blood feeding, while the anterior midgut of the control insects presented normal appearance and volume, and a thin pink cuticle relative to the new exoskeleton was already formed under the abdomen (indicating that these insects were entering in the ecdysis process), the anterior midgut of HSP70 knockdown insects was still full of blood, occupying virtually all the abdominal cavity and there was no sign of a new cuticle formation (Fig. 13.1).

The permanence of most of the blood proteins in the anterior midgut over the time after feeding indicated that the blood transport to the posterior midgut was greatly reduced in HSP70 knockdown insects. From the 10th day after feeding, there was a significant reduction in the amount of total proteins in the AM of control insects, which normally passed to PM to be digested. The HSP70 knockdown in

Aedes aegypti mosquitoes showed similar results and the total protein levels in the AM remained high for a long time after blood-feeding, indicating that blood processing and digestion was impaired in knockdown mosquitoes, with 25% reduction in egg production (Benoit et al. 2011).

The impairment of digestion in HSP70 knockdown *R. prolixus* was clearly demonstrated by reduction in the protease activity in the posterior midgut. Among the 3rd and the 14th day after feeding, a significant increase occurs in the endoprotease activity in the posterior midgut of control insects, while in HSP70 knockdown insects, the protease activity exhibited levels close to zero.

In addition to the digestion process, the post-feeding respiratory metabolism of HSP70 knockdown insects was also disturbed. In insects, the gases exchange between the atmosphere and metabolically active tissues is accompanied by air filling in the tracheal system, which consists of spiracles, trachea, and tracheoles. Spiracles are external openings that act as valves present in the abdomen and in the thorax of the insects, and tracheal trunks that are subdivided into smaller tracheas are found in the inner part (Contreras and Bradley 2010). Monitoring insects in a respirometer can generate basically three different breathing patterns. The discontinuous pattern is characterized by three well-defined phases: a phase in which the respiratory spiracles are completely open and the gases are free to diffuse between the insect tracheal space and the external atmosphere, a phase in which the spiracles are completely closed, and another phase in which the spiracles open and close quickly. This last respiratory pattern has been observed in several insects, including *R. prolixus*, in situations where the metabolic rate is low (when they are not moving or not fed), and seems to be important to avoid unnecessary water loss during respiration. In the cyclic pattern, peaks of CO_2 release occur with a certain regularity, however, between the peaks, the respiratory spiracles never close completely and the CO_2 release does not reach zero. This respiratory pattern can be interpreted as an oscillation between the phase in which the spiracles are fully opened and a phase of reduced CO_2 release. In the continuous respiratory pattern, CO_2 release is continuous and there is no period of total closure of the spiracles. Cyclic and continuous respiratory patterns are usually observed in situations of high energy costs (Contreras and Bradley 2009).

R. prolixus bugs monitored in a respirometer during feeding on an artificial feeder (blood heated at 37 °C) presented a continuous respiratory pattern, keeping the spiracles open or partially open during the whole feeding period, which is a high energetic cost for the insect metabolism. The respiratory pattern during *R. prolixus* nymphs blood ingestion is quite similar to the breathing pattern observed by Contreras and Bradley (2009), when this same triatomine was monitored in the respirometer during continuous exposure at a temperature of 35 °C.

From 72 h after blood feeding, HSP70 knockdown insects presented an altered energetic metabolism. Normally, *R. prolixus* nymphs maintained their typical cyclic breathing pattern after blood feeding. In contrast, insects that were silenced for HSP70 can maintain their cyclic respiratory pattern, but with a much lower frequency of CO_2 release peaks or altered their profile to a continuous respiratory pattern with no clear peaks of CO_2 release. The rate of CO_2 production by the control

insects increased significantly 72 h after feeding, probably due to the beginning of the blood processing and digestion, processes that require an increase in the metabolic rate, while in HSP70 knockdown insects, the amount of CO_2 produced was significantly reduced, indicating a need for energy savings in these insects, which is probably a consequence of the various physiological disorders that appear to occur in these insects. In hematophagous flies of the genus *Glossina*, Taylor (1977) observed that there was a correlation between the amount of blood ingested during feeding and oxygen consumption, and suggests that the high metabolism of these insects after feeding probably reflects the energy demand for the digestion process in these flies.

In *R. prolixus*, lysozyme expression in the midgut tends to increase more than 500-fold after blood feeding in normal conditions, while in HSP70 knockdown insects, lysozyme transcripts appeared reduced to levels close to zero. Lysozyme is an important enzyme that catalyses the hydrolysis of glycoside bonds of the peptidoglycan layer present in the cell wall of some bacteria, causing the lysis of these bacterial cells (Kollien et al. 2003). In addition to provide protection against these microorganisms present in the environment, lysozymes also act in the digestion of symbiotic bacteria, which develop at high densities in the anterior midgut after blood (Ribeiro et al. 2014). Although lysozyme appeared more expressed in anterior than in posterior midgut, it is in the posterior midgut that most symbionts are digested, mainly because lysozyme acts better in slightly acidic pH (Balczun et al. 2008). It has been demonstrated that lysozyme expression can be activated in the medium intestine of *R. prolixus* through the injection of bacteria or through *T. cruzi* feeding (Ursic-Bedoya et al. 2008).

The temporal analysis of the lysozyme expression at different periods before and after feeding indicated that the blood supply induces an increase in lysozyme expression in the midgut of *R. prolixus* 5th instar nymphs, with a peak approximately 5 days after feeding, while starved insects presented a basal expression of this enzyme. Kollien et al. (2003) reported that the peak of higher expression of intestinal lysozyme in *Triatoma infestans* is on the 15th day after feeding. This high expression of lysozyme on the 15th day after feeding may be related to the process of ecdysis, which coincides with this time. In this situation, lysozyme could act as a protection for the insect, which during the exoskeleton exchange may be more susceptible to infection by environmental bacteria.

After the blood ingestion, an immune response is stimulated in *R. prolixus* midgut. The expression of genes related to relish, IMD, Defensine and Lysozyme were up-regulated after blood feeding (Paim et al. 2016). In addition to ecdysteroid control, the blood meal components are important to *R. prolixus* immune response induction (Azambuja et al. 1997). On the other hand, in HSP70 knockdown bugs, the immune response was impaired, once immune genes related to lysozyme, immune deficiency (IMD) and Relish were not up-regulated following the blood meal, as if the insect had not responded to the arrival of blood in the intestinal tract.

In blood-sucking arthropods midgut, blood meal induces bacterial proliferation. The activation of immune responses in insects depends mainly on two intracellular immune cascades: the Toll and the IMD pathways (Vieira et al. 2014). Relish and

IMD are molecules involved in the IMD pathway. Relish is a transcription factor that induces antimicrobial peptide expression, especially against gram-negative bacteria. Lysozymes participate in the hydrolysis of the bacterial cell wall peptidoglycans, preventing the colonization of the digestive tract by pathogens, in addition to also have a digestive function in triatomine bugs. The up-regulation of these antimicrobial genes may be important to control the bacterial population that may develop in high densities on the midgut after a blood meal.

13.2 Conclusions

Haematophagy has independently evolved many times among arthropods. Yet, feeding on blood requires specific adaptations to cope with the many risks and stressors associated to feeding on the blood of endothermic vertebrates. Heat Shock Proteins play a crucial role in protecting the physiological integrity of blood-sucking animals at each feeding event. The level of different HSP significantly increases during the hours following the intake a blood meal, constituting a measure of molecular reparation. Their manipulative reduction also impacts several physiological processes, such as blood-digestion, moult, metabolism and even survival.

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Chapter 14
Heat Shock Proteins in Leptospirosis

Arada Vinaiphat and Visith Thongboonkerd

Abstract Leptospirosis caused by pathogenic *Leptospira* spirochetes remains an important zoonotic disease worldwide. Like many other dimorphic bacterial pathogens, leptospires have abilities to adapt themselves to survive in a wide range of environmental conditions outside and inside the infected hosts. Recent investigations using genomics and proteomics approaches have revealed that several heat shock proteins (HSP) encoded by the conserved immunodominant antigenic region of *Hsp* genes among the pathogenic leptospires are associated with their adaption to survive, infectivity and virulence. Understanding how HSP are differentially expressed and regulated during leptospiral infection is thus crucial to develop better serodiagnostic test and vaccine for clinical use. This chapter summarizes the current knowledge of HSP expression and their roles in leptospirosis and also extensively discusses future perspectives of this arena to battle leptospirosis with the ultimate goals for better therapeutic outcome and successful prevention.

Keywords Chaperone · HSP · Immunoproteomics · Leptospira · Leptospirosis · OMPs · Proteomics · Virulence

Abbreviations

APC	Antigen presenting cells
HSP	Heat shock proteins
LPS	Lipopolysaccharide
MAT	Microscopic agglutination test
MHC	Major histocompatibility complex
MS	Mass spectrometry
OMPs	Outer-membrane proteins
sHSP	Small heat shock proteins

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14.1 Introduction

Leptospires are spirochetes, a group of bacteria that were diverged early in bacterial evolution and are one of a few assigned to their own monophylectic clade, distinct from other bacterial phyla (Paster et al. 1991). Most leptospires are highly motile, slow-growing, obligate aerobes with an optimal growth temperature of 30 °C, and are morphologically distinguishable from other spirochetes by their unique hook-shaped ends (Picardeau 2017). With recent advancements, whole-genome sequencing of several *Leptospira* species has been accomplished, confirming their unique physiological and pathogenic features (Bulach et al. 2006; Picardeau et al. 2008; Ren et al. 2003). The genome of the pathogenic leptospires consists of two circular chromosomes and is considerably larger when compared to other bacteria of the spirochete phylum. This difference in the genome size means that average protein-coding contents of the pathogenic leptospires are generally greater than other spirochetes, reflecting their ability to occupy and survive in a wide range of habitats even with a drastic shift from host physiological condition to external environments (Bharti et al. 2003; Boursaux-Eude et al. 1998).

Species classification of the *Leptospira* genus is based on DNA sequence relatedness (Brenner et al. 1999). Although this recently developed genetic classification has become an alternative approach to the conventional antigenic classification, the conventional system is still more desirable among clinicians and epidemiologists for clinical diagnostics and research purposes (Terpstra 1992). For this reason, both classification systems are being used in conjunction. Typically, leptospires can be categorized into "pathogenic" (*L. interrogans, L. kirschneri, L. borgpetersenii, L. santarosai, L noguchii, L. weilii, L. alexanderi, and L. alstonii*) and "saprophytic" (or "non-pathogenic") (*L. biflexa, L. wolbachii, L. kmetyi, L. vanthielii, L. terpstrae, and L. yanagawae*) groups. Among these, *L. interrogans* is the most common and well recognized species and contains the highest number of pathogenic serovars. Another class, namely "intermediate/opportunistic" group, that has been deciphering contains species of unclear pathogenicity (*L. inadai, L. broomii, L. fainei, L. wolffii, and L. licerasiae*) (Bourhy et al. 2014; Fouts et al. 2016; Saito et al. 2013). Through years of extensive serological investigations, more than 300 leptospiral serovars have been defined and reported (Lane and Dore 2016).

In general, the genetics and molecular compositions of leptospires can determine their pathogenicity and virulence (Xu et al. 2016). Recent whole-genome sequencing of the pathogenic species *L. interrogans* (Nascimento et al. 2004; Ren et al. 2003) and *L. borgpetersenii* (Bulach et al. 2006) and the saprophytic species *L. biflexa* (Picardeau et al. 2008) has revealed differential genome expression among them and a set of genes that reflect the very different lifestyles of these two groups of leptospiral species (Adler and de la Pena 2010). For example, out of 4768 genes identified in the pathogenic species *L. interrogans*, at least 50 genes are related to motility. This finding explains the ability of *Leptospira* to swim through viscous solution/media/environment to migrate through barriers of host tissues at the initial entry site and to disseminate to the target organs (Ren et al. 2003). In addition, sev-

eral genes encoding specific virulence-related proteins have been exclusively identified in the pathogenic species, underscoring the role of differential genome in the pathogenicity and virulence of leptospires (Xu et al. 2016).

Despite the fact that leptospiral agents have been discovered for over a century, their detailed biology and precise pathogenesis remain vaguely defined. Continuing efforts to define precise cellular and molecular mechanisms of leptospirosis are still needed to accomplish the ultimate goals to improve treatment and diagnostics and to develop effective prophylactic strategy.

14.2 Roles of HSP in Pathogenic and Saprophytic Leptospires Upon Heat Stress

Temperature shift has been reported to alter HSP synthesis in leptospires. Changes in expression of HSP in response to temperature shift (from 30 °C to 47 °C) have been investigated in model organisms of the pathogenic *L. interrogans* serovar Harjo and the saprophytic *L. biflexa* serovar Patoc, as well as other spirochetal species (Stamm et al. 1991). The data obtained from [^{35}S]methionine-labeled proteins of these spirochetal species, before and after heat shock, has shown that *Leptospira* species have the highest number of the altered HSP as compared to other spirochetal species (Stamm et al. 1991). This information may explain the nature of leptospires that can persist in a wide range of environmental temperature fluctuations and changes in expression of HSP may be responsible for such adaptation to survive.

Interestingly, additional reference has shown that the non-pathogenic *L. biflexa* synthesizes greater number of discernible HSP than the pathogenic *L. interrogans* (19 vs. 7 HSP, respectively). Moreover, *L. biflexa* contains a greater number of genes involving in cellular processes and cell signaling (Picardeau et al. 2008). These findings can be explained by the current genomic studies. Generally, differential genome expression and contents between organisms are correlated with their biological differences. The acquisition of larger pool of genes encoding proteins involving in posttranslational modifications, protein turnover and chaperone reflects the plasticity of proteins required for the survival in the wide spectrum of ecological niches where nutrient deprivation or temperature shift may be continuously encountered (Adler et al. 2011; Picardeau et al. 2008). Thus, the greater number of HSP in *L. biflexa* mey be responsible for long-term environmental persistence of the free-living saprophytic leptospires.

Another genome analysis has revealed that the genome of *L. interrogans* is larger than that of *L. biflexa* due to frequent DNA rearrangements and insertion. The extended genetic contents of *L. interrogans* presumably occur for the survival of extended passage during transmission cycle from animal hosts to aquatic environments, in contrast to a minimal rearrangement of *L. biflexa* genome that has restricted transmission potential (Nakamoto et al. 2014). Nevertheless, this raises a question that why such large genome does not lead to greater expression of HSP in the patho-

genic leptospires as they are chaperones that aid in stabilizing expression of downstream signaling cascade required to adapt to various environmental conditions encountered upon infection. It is likely that certain leptospiral HSP expressed during infection may or may not be expressed *in vitro* and that different bacterial strains may express HSP at different time-points upon heat induction. Nevertheless, this still unclear phenomenon deserves further elucidations.

The expression of two highly conserved HSP, DnaK and GroEL (members of the Hsp60 and Hsp70 families, respectively), in leptospires has been evident. Both of them have been successfully identified in *L. interrogans* and *L. biflexa*, as well as in other spirochetes such as *Treponema pallidum*, *T. phagedenis*, *T.denticola*, and *Borrelia burgdorferi*. Interestingly, although DnaK is present in *T. pallidum* and *T. denticola* and GroEL is present in *B. burgdorferi,* their expression levels are not significantly changed following temperature shift comparing to the basal state. This indicates that not all spirochetes can exhibit thermo-inducible HSP (Cluss and Boothby 1990; Stamm et al. 1991). The ability for leptospires to possess and synthesize HSP that exhibit true heat shock response suggests their important roles, specifically in *Leptospira* species. This notion is also supported by another discovery demonstrating that the reactivity of GroEL found in other non-*Leptospira* is considerably weaker than that detected in *Leptospira* serovars (Park et al. 1999).

Temperature regulation of small HSP (sHSP) has been also demonstrated in leptorpires. Hsp15, a member of the Hsp20/α-crystallin family, is the first of spirochete sHSP reported. An initial molecular study using genomic approach has demonstrated the presence of *hsp15* gene in six different serovars of the pathogenic *L. interrogans*, whereas none is detected in the saprophytic *L. biflexa* and *B. burgdorferi* (Nally et al. 2001a). Additionally, induction of Hsp15 protein synthesis takes place within hours after temperature shift (from 30 °C to 37 °C) in *L. interrogans* using Hsp15-specific antiserum to detect Hsp15 protein from whole cell lysate (Nally et al. 2001a).

Taken together, various HSP are the important constituents in spirochetes. The differential expression of HSP in certain *Leptospira* serovars implicates that thermo-inducible HSP are strain-specific and the adaptive response of HSP during temperature shift may play specific roles in infectivity and virulence of the pathogenic species/serovars.

14.3 Roles of HSP in Immunology and Pathology of Leptospiral Infection

Many infections have been demonstrated to be associated with HSP. Due to their high expression levels during heat and other stresses it is not surprising that these proteins are likely to be processed by antigen presenting cells as major foreign antigens for presentation to lymphocytes. In fact, HSP have been reported as

immunodominant antigens recognized by humoral antibodies and activated T cells, inducing very strong humoral and cellular immune response in many infections, as diverse as tuberculosis (by *Mycobacterium tuberculosis*), leprosy (by *M. leprae*), malaria (by *Plasmodium falciparum*), Q fever (by *Coxiella burnetti*), Chagas disease (by *Trypanosoma cruzi*), and Leishmaniasis (by *Leishmania major*) (Lindquist and Craig 1988). Although the genetic system of HSP and their molecular entities vary between organisms, the overall molecular functions of these chaperones are somewhat universal (Ballard et al. 1993; Ballard et al. 1998). This is also the case for leptospirosis. Using sera from patients with leptospirosis during an urban epidemic in Brazil, protein antigens derived from a clinically isolated *L. interrogans* serovar Copenhageni have been reported (Guerreiro et al. 2001). Out of 13 antigens most commonly expressed, GroEL and DnaK are two of the targets for humoral immune response during the natural course of leptospiral infection. The seroreactivity of anti-GroEL and anti-DnaK during both acute and convalescent phases of infection are the second and third best seroreactivities (after the first being LipL32, the major outer membrane lipoprotein). This strongly indicates that these two HSP are immunodominant antigens (Guerreiro et al. 2001).

Moreover, GroEL and DnaK are conserved in all pathogenic *Leptospira* species investigated, including *L. interrogans, L. kirschneri, L. borgpetersenii, L. noguchii, L. santarosai, L. weilii,* and *L. inadai* (Guerreiro et al. 2001; Lindquist and Craig 1988). However, GroEL and DnaK may not be the only two HSP that serve as immunoreactive proteins or antigens. Other three unidentified small protein antigens have been also observed (due to the lack of specific antibodies at that time, they remain uncharacterized) (Guerreiro et al. 2001; Lindquist and Craig 1988). Perhaps, one of these low molecular weight proteins may potentially be a member of sHSP family because one of the sHSP, Hsp15, has been later reported to immunoreact with Hsp15-specific antibody derived from naturally infected convalescent mares' sera (Nally et al. 2001a).

Even with the aforementioned evidence, molecular mechanisms for HSP in the pathogenesis of leptospirosis remain poorly understood. On the other hand, the presence of HSP can provide adaptive and survival advantages for bacteria, enhancing their ability to infect hosts and ultimately cause disease. Paradoxically, leptospiral HSP expressed during infection can trigger host immune response. Through collective HSP expression data, it has shown that shifting temperature from 30 °C to 37 °C can cause up-regulation of HSP (i.e. GroEL, DnaK, and Hsp15) of *L. interrogans*, whereas prolonging their growth for several days (to mimic conditions during the infection) can suppress their expression and synthesis to basal levels (Nally et al. 2001a, b; Qin et al. 2006). This suggests that HSP may participate in leptospiral pathogenesis at an early stage of the infection, but their down-regulation at a later phase might be from host immune response or adaptation of leptospires themselves to avoid being detected by the host immune system (Nally et al. 2001a, b; Qin et al. 2006). Precise mechanisms underlying this phenomenon should be further investigated.

14.4 Investigations of HSP by High-Throughput Mass Spectrometry (MS)-Based Proteomics and Immunoproteomics

A number of genomic analyses of various *Leptospira* strains have provided insights into regulatory mechanisms of bacteria in response to environmental stimuli and their ability to survive within hosts (Nascimento et al. 2004; Ren et al. 2003; Sauer et al. 2004). Tremendous progress has been made to define genes that are differentially expressed in pathogenic vs. non-pathogenic *Leptospira* species, and among the pathogenic *Leptospira* species/serovars grown under experimental interventions to simulate *in vivo* conditions within hosts, e.g., temperature shift, low iron state, and in the presence of serum (Adler et al. 2011; Bulach et al. 2006; Picardeau et al. 2008; Qin et al. 2006). Additionally, genomic approaches have led to identification of genes potentially encoded virulence-associated proteins. However, gene expression alone can neither determine how particular biological processes are regulated, nor reflect differences among cellular states (Cox and Mann 2007). As such, a comprehensive comparative study on changes in the final gene products (i.e., proteins) is required to better understand changes or adaptive processes of bacteria during/after infection.

Advances in understanding the pathogenesis of leptospirosis has been evolved around the use of the high-throughput MS-based proteomics and immunoproteomics approaches (Aebersold and Mann 2003; Thongboonkerd 2008). One of the advantages of using MS-based proteomics and immunoproteomics approaches to detect proteins and antigens, respectively, is that specific antibodies to leptospiral proteins are not required. Another advantage is that these advanced approaches offer opportunities to discover new or previously unknown proteins/antigens all the time (Aebersold and Mann 2003; Thongboonkerd 2008). The recent knowledge of MS-based proteomics/immunoproteomics applications for characterizing HSP and identifying candidate virulence factors are summarized and discussed below.

14.4.1 Cytosolic HSP

Recent studies using MS-based proteomics to examine leptospiral proteome have allowed feasible identification of differential HSP expression among different pathogenic and non-pathogenic *Leptospira* species and serovars (Thongboonkerd 2008). The results show a multidimensional view of the regulation of HSP expression in various leptospiral species/serovars. Comparative analysis of the two model organisms of pathogenic *L. interrogans* and non-pathogenic *L. biflexa* using MS-based proteomics has permitted the identification of a novel Hsp homolog (Clp ATPase), which is a member of the Hsp100 family that has not been previously detected using conventional immunoblotting (Thongboonkerd et al. 2009). By comparing HSP expression among the four pathogenic *L. interrogans* serovars (Australis,

Bratislava, Autumnalis, and Icterohaemorrhagiae), the most virulent serovar Icterohaemorrhagiae has the greatest levels of HSP, including DnaK, GroEL, and ATP-dependent Clp protease proteolytic subunit, underlining the notion that DnaK, GroEL, and Clp may serve as the pathogenic factors (Kositanont et al. 2007; Thongboonkerd et al. 2009).

Another differential quantitative proteome analysis of pathogenic *L. interrogans* serovar Copenhageni cultured under limited amounts of iron and serum to simulate *in vivo* host environment has immensely expanded the current knowledge of HSP dynamicity in the context of leptospiral pathogenesis (Eshghi et al. 2009). Upon exposure to the *in vivo*-like environmental conditions, the overall decrease in leptospiral protein expression has been observed. These decreased proteins also include three HSP, i.e., IbpA-1, GroES (19-kDa chaperonin) and ClpA-1 ATP-dependent protease (Eshghi et al. 2009). These data are in concordance with those reported in previous studies showing that HSP are up-regulated during an early phase of leptospiral infection but subsequently down-regulated at the later phase (Eshghi et al. 2009; Nally et al. 2001b). The decrease in HSP levels at this time-point is correlated with bacteremic or septicemic phase of leptospiral infection that usually lasts from three to seven days (Adler and de la Pena 2010).

14.4.2 Outer Membrane HSP

Spirochaetes is a distinct phylum of bacteria with a unique double-layered membrane architecture (Haake 2000). Proteins localized at outer membrane of leptospiral cells are particularly important for bacterial colonization and intracellular survival in hosts as these proteins are at the site of interaction with host epithelial cells and immune system (Ghazaei 2017). Characterizations of outer-membrane proteins (OMPs) are thus crucial to better understand mechanisms underlying bacterial colonization in hosts.

GroEL has been firstly identified on the outer membrane of *L. kirschneri* using surface-selective biotinylation followed by MS for protein identification (Cullen et al. 2005). The low level of GroEL among the identified OMPs has been once thought to be from contamination of the highly abundant cytoplasmic GroEL during sample preparation (Cullen et al. 2005). Subsequent MS-based proteomics studies have also identified GroEL on the surface of *Leptospira*, confirming its existence among OMPs (Natarajaseenivasan et al. 2011). The significant role of the surface GroEL as a potential virulence factor has been also addressed. Immunoreactivity of the recombinant surface GroEL of *L. interrogans* serovar Autumnalis and leptospirosis patients' sera with different clinical entities (93 with pulmonary involvement, 121 with renal failure,137 pediatric patients, and 93 pregnant cases) has been shown to be highly sensitive (90.6%) and specific (94.9%) (Natarajaseenivasan et al. 2011). Such highly immunoreactive nature of GroEL that reacts to a wide range of sera from patients with different clinical manifestations may be helpful for the disease diagnostics.

Indeed, HSP are predominantly localized in the cytoplasm. The exact mechanism that induces translocation of cytosolic HSP to the out membrane remains unclear as it lacks specific leader sequence required for translocation and targeting to the membrane. Nevertheless, expression of several HSP on the outer membrane has been shown to be inducible by temperature increase. Altered surface expression of *L. interrogans* serovar Lai after an overnight exposure to heat stress (37 °C) has been reported, including increased levels of HSP (IbpA-1, IbpA-2 (Hsp15), DnaK, GroEL, GrpE and ClpA) (Lo et al. 2009). In *E. coli*, sHSP act as an integral part of the DnaK/DnaJ/GrpE and GroEL/GroES chaperone systems by stabilizing these complexes during stress (Huesca et al. 1998). Hence, the remarkable increases in small HSP and various HSP on bacterial cell surface upon overnight temperature upshift are consistent with the concept that HSP may play major role(s) in the early phase of infection by stabilizing multi-chaperone network to maintain/protect bacterial membrane structural and functional integrity during thermal stress.

Although it is still too early to assume that leptospiral surface HSP take part in host tissue invasion (i.e., enhancing/assisting bacterial attachment onto host cell surface), several lines of recent evidence have demonstrated that surface DnaK of bacterial cells (i.e., *Helicobacter pylori* and *E. coli*) have the ability to act as a ligand, binding to the receptor on the host cell surface (Ghazaei 2017; Huesca et al. 1998). Knockout of *DnaK/DnaJ* operon in *Salmonella enterica* and *Campylobacter jejuni* results in the loss of ability of pathogens to colonize within the host tissue, whereas restoration of these genes can restore their invasive function (Genevaux et al. 2004). Moreover, the infectivity of certain groups of bacteria can be improved when they express HSP (not limited only to DnaK) or other co-chaperones (Ghazaei 2017; Sikora and Grzesiuk 2007). Despite these correlations, the direct attachment of leptospiral surface HSP to host cells as well as their contribution to the pathogenesis remains to be proven. Moreover, a growing number of surface HSP are highly expected. Further MS-based proteomics analyses of surface HSP among pathogenic and non-pathogenic serovars at different stages of infection or upon various stresses may bridge the information gap of why particular serovars can invade hosts better than others.

14.5 HSP as Diagnostic Markers in Acute Leptospirosis

Several methods, i.e., general laboratory tests, microscopy, cultivation, PCR-based molecular technique, and serology are currently available for diagnosis of leptospirosis (Sayyed Mousavi et al. 2017). Among these, serological test is the most frequently used technique (Bharti et al. 2003). Microscopic agglutination test (MAT) that uses a panel of live leptospires isolated from the area where patients become infected to detect antibodies in patients' sera has been considered as the gold standard method due to its high specificity. However, specialized techniques and skills

to maintain a large batch of live leptospires are required, thus restricting the use of MAT to only some reference laboratories (Goris and Hartskeerl 2014). Other currently available serological assays, i.e., macroscopic agglutination (Brandao et al. 1998), indirect hemagglutination (Levett and Whittington 1998) and ELISA (Winslow et al. 1997), have shown reasonable specificities (89–95%) but with unacceptable sensitivities (<72%) (Riazi et al. 2014). Therefore, development of better diagnostic tests for rapid and accurate diagnosis should be done.

Specific diagnosis of leptospirosis is still a challenge due to a high degree of cross-reactivity between different serovars. Ideally, the best immunodominant antigens for serodiagnostic test are the ones that are highly conserved among diverse pathogenic leptospiral strains. Efforts have been made to apply leptospiral lipopolysaccharide (LPS) as a candidate antigen for serodiagnosis due to its high sensitivity and specificity (Priya et al. 2003). However, its antigenic variability among leptospiral serovars has limited its use (Adler and de la Pena 2010; Matthias and Levett 2002).

Several recent studies have demonstrated the potential use of DnaK and GroEL as diagnostic markers in leptospirosis. DnaK has been found to immunoreact with sera of patients with leptospirosis in both acute and convalescent phases with a high seroreactivity, suggesting that DnaK is one of the major target antigens for humoral immunity (Riazi et al. 2014). The same finding has been also obtained for GroEL (Park et al. 1999). However, subsequent study has pointed out that seroreactivities of these HSP can be also found in 7–23% of the controlled sera from blood bank donors or healthy individuals, reflecting the ubiquitous expression of both proteins in both pathogenic and non-pathogenic *Leptospira* and may be other bacteria (Guerreiro et al. 2001). The fact that IgG response in the controlled group appears positive against leptospiral GroEL and DnaK antigens has suggested high degree of cross-reactivity from other bacteria, limiting the feasibility of using GroEL or DnaK as specific markers for leptospiral serodiagnosis (Guerreiro et al. 2001). This data also points out that other HSP that expresses during infection and exhibits less degree of conservation may serve as the better candidates for being used as markers for serodiagnosis of leptospirosis.

Despite high degree of cross-reactivity of IgG in the controlled sera to GroEL derived from *Leptospira* whole cell lysate, different scenario has been shown when recombinant surface GroEL is used. The study has shown that only 1.0–3.5% of sera from controls infected with other bacteria immunoreact with such recombinant surface GroEL, with sensitivity and specificity of 91.0–94.6% (Natarajaseenivasan et al. 2011). Possible explanation for these discrepancies between using whole cell lysate and recombinant surface GroEL is that recombinant surface HSP may contain extra sequence for translocation; thereby possess different immunogenicity as compared to their cytoplasmic forms. From these results, the immunogenic surface HSP clearly offer potential use in the serological tests for early diagnosis of leptospirosis, though precise mechanism for better outcome of the recombinant surface HSP remain to be elucidated.

14.6 HSP for Vaccine Development

One of the major challenges in leptospirosis research is vaccine development for its prevention. To date, there are no vaccines universally available for leptospirosis due to variations of *Leptospira* serovars (Bharti et al. 2003). In general, leptospiral vaccines that consist of inactivated whole-cell immunogens rely mostly on the immunorecognition of carbohydrate epitopes of LPS (Guerreiro et al. 2001). However, this LPS-mediated immunity is limited to homologous serovars, thereby does not give cross-protection against serovars not formulated in the vaccine (Guerreiro et al. 2001). Other leptospiral surface-exposed immunogens, OMPs, in combination with LPS have been demonstrated to provide better protective effects and fewer side effects (Evangelista and Coburn 2010; Haake et al. 1999; Yan et al. 2009). However, such approach is still limited to only certain serovars and does not induce long-term protection against leptospiral infection. The better vaccine that elicits an effective cross-protection against *Leptospira* serovars is therefore urgently required.

HSP are being considered as candidate targets for the development of vaccines because of their high conservation among diverse leptospiral serovars. Several studies have suggested the use of HSP, specifically DnaK, as one of the constituents in vaccines due to an exceptional degree of immunogenicity and its known ability to enhance the major histocompatibility complex (MHC) antigen processing and presentation of antigen presenting cells (APC) to T-lymphocytes (Barrios et al. 1992; Tobian et al. 2004; van Eden et al. 2003). In fact, genetic fusion of DnaK with four leptospiral OMPs (Lsa21, Lp95, rLIC10494, and rLIC12730), which involves host-bacterial interaction, elicits greater humoral and cell-mediated immune responses in mice than using individual proteins (Atzingen et al. 2014). These important findings strengthen the use of leptospiral HSP in conjunction with OMPs for development of new vaccines against leptospirosis.

14.7 Conclusions and Future Perspectives

The expression of various HSP in *Leptospira* has been identified under both physiologic and pathologic conditions. Recent studies of HSP and their expression patterns in leptospires have advanced our understanding of the biological significance of HSP as molecular chaperones aiding in adaptive and survival processes that affect their infectivity and virulence. Despite these convincing data, several aspects of HSP remain to be further elucidated. During the past few years, a growing number of studies using proteomics and immunoproteomics approaches have led to the increased number of potential leptospiral immunogens built in library. Nevertheless, putative immunogens, particularly HSP, seem to be underrepresented. Proteome profiling using other recently developed methodologies can be also used as the alternative means to achieve the goals (Thongboonkerd 2008). These techniques can help unraveling expression of HSP and promoting coherent results. Also, a

comprehensive systematic analysis of HSP expression in multi-aspects is required. The expression of HSP, particularly in *Leptospira*, is considerably dynamic than other organisms. Unique mechanism of genetic regulation of leptospires with more than 300 serovars coincides with the differences in clinical presentations observed in infected patients (Adler et al. 2011). Therefore, defining alterations in HSP expression among pathogenic, non-pathogenic, and/or intermediate strains in response to different stimuli or at different stages of infection is needed (because differential patterns of HSP may account for the observed phenotypic differences among these strains (Thongboonkerd 2008; Thongboonkerd et al. 2009)).

The clinical features of leptospirosis vary depending on both pathogen factors and host response (Lane and Dore 2016). Currently, the role of host HSP in immune system against *Leptospira* has not yet been addressed. Certain HSP reside in APC have been demonstrated to be involved in many aspects of the immune response, i.e., antigen presentation and cytotoxic cell killing of the immune targets (Kiang and Tsokos 1998). More importantly, although the role of HSP in autoimmune disease is not well supported, there are a number of studies that have reported a strong binding preference of T-cell receptor (αβ-positive) against an epitope of self HSP65, suggesting a very likely event of autoimmune response elicited by HSP (Anderton et al. 1995). Considering these relations, using HSP as one of the constituents in vaccine may stimulate adaptive immune response that recognizes not only pathogens, but also self HSP. Therefore, understanding the function of host HSP as the regulator of the immune response to leptospires is definitely valuable during vaccine design and development.

Most studies on HSP have been aimed to define novel immunogens with potential use in diagnostics and vaccine development. Less attention has been given to the investigation of molecular mechanisms that HSP contribute to the pathogenesis of leptospirosis. So far, the association of HSP with leptospiral pathogenesis and virulence has been explained based mostly on assumption from their known functions in other model organisms. However, different organisms especially the ones that have evolved independently (i.e., leptospires) certainly must have diverse gene/protein regulatory processes. Understanding the sophisticated function of leptospiral HSP may provide a better view of the pathogenic mechanisms as well as pathophysiology of leptospirosis for better therapeutic outcome and successful prevention.

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Chapter 15
Heat Shock Proteins in Vector-pathogen Interactions: The *Anaplasma phagocytophilum* Model

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Abstract *Anaplasma phagocytophilum* is an emerging zoonotic pathogen that is transmitted by *Ixodes* ticks and causes human granulocytic anaplasmosis. Several recent studies have shown that tick infection by *A. phagocytophilum* induces complex changes mediated by different mechanisms such as remodeling of cytoskeleton, inhibition of cell apoptosis, modification of metabolism and cell epigenetics, manipulation of the immune response and stress response. In particular, heat shock proteins (Hsp), a group of highly conserved proteins, play an important role in tick-pathogen interactions. Not only tick Hsp mediate the response to *A. phagocytophilum* infection, but also bacterial Hsp bind tick and vertebrate host cells. Herein, we reviewed the literature and provided new insides on the role of bacterial and tick Hsp in tick-pathogen interactions. We combined the analysis of published data on genomics, transcriptomics and proteomics of the response of *Ixodes scapularis* ticks to *A. phagocytophilum* infection. In addition, functional studies were conducted

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to test some of the hypotheses on the role of tick Hsp in response to *A. phagocytophilum* infection. These results provide a more comprehensive view of the major Hsp involved in the response to pathogen infection in ticks.

Keywords *Anaplasma phagocytophilum* · Apoptosis · Heat shock proteins · *Ixodes scapularis* · Tick-borne diseases · Vaccine · Vector-pathogen interaction

Abbreviations

HGA	Human granulocytic anaplasmosis
HIF-1α	Transcription factor hypoxia inducible factor 1 alpha
HLHsp70	*Haemaphysalis longicornis* Hsp70
Hsp	Heat shock proteins
LIV	Louping-ill virus
ML	Maximum likelihood
MSP4	Major surface protein 4
Ra-sHSPI	sHsp from *Rhipicephalus annulatus* ticks
sHsp	Small Hsp
T4SS	Type 4 secretion system
TBEV	Tick-borne encephalitis virus

15.1 Introduction

Ixodes scapularis ticks are blood-feeding arthropods that transmit pathogens such as *Borrelia burgdorferi* (Lyme disease) and *Anaplasma phagocytophilum* (human granulocytic anaplasmosis; HGA) (de la Fuente et al. 2017). The infection and colonization of ticks by the obligate intracellular bacterium, *A. phagocytophilum* (Rickettsiales: Anaplasmataceae) occurs first in midgut cells and then subsequently in other tissues including hemocytes and salivary glands from where transmission occurs during feeding (Severo et al. 2015). Recent results have shown that *A. phagocytophilum* infection of ticks induces complex changes mediated by different mechanisms that include but are not limited to remodeling of the cytoskeleton, inhibition of cell apoptosis, manipulation of the immune response, and modification of cell epigenetics and metabolism (Ayllón et al. 2015; Villar et al. 2015a; de la Fuente et al. 2016a, 2017; Cabezas-Cruz et al. 2016, 2017a). Ticks respond to infection by activating alternative pathways to regulate cell apoptosis, immunity, metabolism and stress response mediated by heat-shock proteins (Hsp) (de la Fuente et al. 2016b).

Hsp are up-regulated in response to various stress conditions including pathogen infection (Schlesinger 1990; Whitley et al. 1999; Bakthisaran et al. 2015; Yu et al. 2015; Zuo et al. 2016; Carra et al. 2017). The interactions of small Hsp (sHsp) with several proteins have important physiological and pathophysiological consequences, and the mechanism(s) involved in the promiscuous substrate interactions and pleotropic functions of sHsp are still not fully characterized (Schlesinger 1990; Vabulas et al. 2002; Bakthisaran et al. 2015; Carra et al. 2017). In mammals, these proteins have been classified into six major families based on their molecular weight (Table 15.1) (Schlesinger 1990; Bakthisaran et al. 2015), and homologs have been identified in the genome of the tick vector, *I. scapularis* (Table 15.1). The results showed that the regulatory element responsible for Hsp gene expression, an inverted repeat of the 5-nucleotide base pair, nGAAn located about 80–150 base pairs upstream of the transcription start site, was invariant in eukaryotes from yeast to human (Schlesinger 1990). Therefore, the presence of this element constitutes the most definitive evidence that the gene encodes a Hsp. However, other regulatory signals are also involved in the regulation of Hsp expression (Schlesinger 1990). Furthermore, the selective translation of Hsp in stressed cells is facilitated by the lack of introns, the presence of 5′-untranslated regions conferring translational efficiency, and regions in the 3′-untranslated region increasing the mRNA stability (Schlesinger 1990). In contrast to eukaryotes, studies in *Escherichia coli* have shown that Hsp genes form a regulon, appearing simultaneously after regulation by an isomer of the σ subunit regulatory element in the bacterial RNA polymerase (Schlesinger 1990).

The function of Hsp have been characterized in mammals and model organisms such as *Drosophila melanogaster* where increase in protein production in response to stress was first described (Tissières et al. 1974; Schlesinger 1990; Vabulas et al. 2002; Teves and Henikoff 2013; Bakthisaran et al. 2015; Morrow et al. 2016; Yu et al. 2015; Zuo et al. 2016; Carra et al. 2017). In general, large Hsp (Table 15.1) function to protect, preserve and recover the activity of various protein complexes, while sHsp (Table 15.1) are more actively involved in protein degradation in response to stress (Schlesinger 1990; Bakthisaran et al. 2015). However, although Hsp are evolutionary highly conserved, little is known about their function in response to stress and pathogen infection in arthropod vectors such as ticks.

This review focuses on the identification and characterization of tick Hsp homologs using the recently published *I. scapularis* genome sequence and assembly (Gulia-Nuss et al. 2016; de la Fuente et al. 2016c), and their putative role during pathogen infection using the *I. scapularis*-*A. phagocytophilum* model. Additionally, functional studies were conducted to test some of the hypotheses on the role of tick Hsp, and the information on *A. phagocytophilum* Hsp was included in the analysis to better understand the role of these proteins during tick-pathogen interactions.

Table 15.1 Mammalian Hsps and homologues in the tick vector *I. scapularis*

Hsps in mammals	Abbreviation	Family	Location	Main Function	Homologs in ticks (accession number)[a]
Large Hsps					
Heat shock protein 90 beta member 1	HSP90B1	Hsp90	Cytosol, nucleus, mitochondria	Part of the steroid receptor complex	ISCW022766
Heat shock protein 90 alpha	HSP90AA1				ISCW014265
Heat shock protein 75 – A	TRAP1A				ISCW024884
Heat shock protein 75 – B	TRAP1B				ISCW009087
Hypoxia up-regulated protein 1	Grp170	Hsp70/ Dnak		Stress tolerance	ISCW012646
Heat shock protein 70 1A	HSP70-1				ISCW017456
Heat shock protein 70 member 1B – A	HSP70-2A				ISCW024910
Heat shock protein 70 member 1B – B	HSP70-2B				ISCW009157
Heat shock protein 70 member 1B – C	HSP70-2C				ISCW011425
Heat shock protein 70 member 5	HSPA5				ISCW017754
Heat shock protein 70 member 8 – A	HSPA8A				ISCW024057
Heat shock protein 70 member 8 – B	HSPA8B				ISCW000190

(continued)

Table 15.1 (continued)

Hsps in mammals	Abbreviation	Family	Location	Main Function	Homologs in ticks (accession number)[a]
Heat shock protein 70 member 9 – A	HSPA9A				ISCW020763
Heat shock protein 70 member 9 – B	HSPA9B				ISCW017192
Heat shock protein 70 member 9 – C	HSPA9C				ISCW020752
Heat shock protein 70 member 12A	HSP70-12A				ISCW012626
Heat shock protein 70 member 14	HSPA14				ISCW006485
Heat shock 70 kDa protein 4L – A	HSPA4A				ISCW019164[b]
Heat shock 70 kDa protein 4L – B	HSPA4B				ISCW019329[b]
Heat shock 70 kDa protein 4L – C	HSPA4C				ISCW018173
Heat shock 70kDa protein 4 isoform a	HSPA4a				ISCW019328[b]
Heat shock protein 72	HSP72				ISCW015267
Heat shock protein 105	HSP105				ISCW016090
Heat shock protein 60 – A	HSP60A	Hsp60/ GroEL		Molecular chaperone	ISCW017824
Heat shock protein 60 – B	HSP60B				ISCW020787

(continued)

Table 15.1 (continued)

Hsps in mammals	Abbreviation	Family	Location	Main Function	Homologs in ticks (accession number)[a]
Heat shock protein 40 (DnaJ1)	DnaJ1	Hsp40/ DnaJ		Chaperone activity, stimulating the ATPase activity of DnaK	ISCW013384
Heat shock protein 40 (DnaJ2)	DnaJ2				ISCW009131
Heat shock protein 40 (DnaJ3)	DnaJ3				ISCW020762[b]
Heat shock protein 40 (DnaJ4)	DnaJ4				ISCW020633
Heat shock protein 40 (DnaJ8)	DnaJ8				ISCW015579
Heat shock protein 40 (DnaJ9)	DnaJ9				ISCW013814
Heat shock protein 40 (DnaJ10)	DnaJ10				ISCW018779
Heat shock protein 40 (DnaJ11)	DnaJ11				ISCW021534
Heat shock protein 40 (DnaJ12) – A	DnaJ12A				ISCW004472
Heat shock protein 40 (DnaJ12) – B	DnaJ12B				ISCW002129
DnaJ homolog subfamily C member 21	DnaJ21				ISCW014018
DnaJ homolog subfamily C member 25	DnaJ25				ISCW019651
Heat shock protein 40 (DnaJ30)	DnaJ30				ISCW008132

(continued)

Table 15.1 (continued)

Hsps in mammals	Abbreviation	Family	Location	Main Function	Homologs in ticks (accession number)[a]
Small Hsps					
Chaperonin 10 – A	sHsp10A	Cpn10/ GroES	Cytosol, nucleus	Molecular chaperone	ISCW017823
Chaperonin 10 – B	sHsp10B				ISCW020786
Heat shock protein beta-1 – A	sHspB1A	Hsp20		Chaperone activity, stabilization of cytoskeleton, anti-apoptotic and anti-oxidant function	ISCW007793
Heat shock protein beta-1 – B	sHspB1B				ISCW017709
Heat shock protein beta-1 – C	sHspB1C				ISCW023475
Heat shock protein beta-5 – A	sHspB5A			Chaperone activity, stabilization of cytoskeleton, cell cycle, cardioprotection, eye lens refractive index, regulation of muscle differentiation, anti-apoptotic function	ISCW020672
Heat shock protein beta-5 – B	sHspB5B				ISCW002513
Heat shock protein beta-5 – C	sHspB5C				ISCW024922
Heat shock protein beta-5 – D	sHspB5D				ISCW023576
Heat shock protein beta-6 – A	sHspB6A			Smooth muscle relaxation, cardioprotection, chaperone activity, anti-apoptotic function	ISCW001762
Heat shock protein beta-6 – B	sHspB6B				ISCW015266
Heat shock protein beta-6 – C	sHspB6C				ISCW024062[b]
Heat shock protein beta-6 – D	sHspB6D				ISCW024054
Heat shock protein beta-6 – E	sHspB6E				ISCW024834[b]
Heat shock protein beta-7	sHspB7			Chaperone activity, myofibrillar integrity	ISCW024717

(continued)

Table 15.1 (continued)

Hsps in mammals	Abbreviation	Family	Location	Main Function	Homologs in ticks (accession number)[a]
Heat shock protein beta-8	sHspB8			Chaperone activity, induction of autophagy	ISCW001763
Ubiquitin C	UBC	Ubiquitin		Facilitates targeting and removal of proteins denatured by stress	ISCW010407
Ribosomal protein L40	RPL40				ISCW020697
Ribosomal protein S27a	RPS27A				ISCW000125

Hsp family members were collected from Schlesinger (1990), Whitley et al. (1999) and Bakthisaran et al. (2015)

[a]Gene homology was assigned based on top BLAST hits against *Homo sapiens* database

[b]Not found in tick transcriptomics and/or proteomics data

15.1.1 Evolution of HSP and Identification of Homologs in the I. scapularis *Tick Genome*

Hsp are highly conserved across taxonomic lineages, but diversity within each Hsp gene family reflects evolutionary gains and losses of Hsp gene copies (Nei and Rooney 2005; Krenek et al. 2013; Nguyen et al. 2016). The Hsp gene families evolved following both concerted (i.e. all member genes of a family evolve as a unit in concert) and birth-and-death (i.e. new genes are created by gene duplication and some duplicate genes stay in the genome for a long time, whereas others are inactivated or deleted from the genome) evolution (Nei and Rooney 2005). In the *I. scapularis* genome we recovered conserved Hsp from all of the major gene families including large (Hsp90, Hsp70, Hsp60, Hsp40) and small (Cpn10 and Hsp20) Hsp (Table 15.1). Several members of Hsp70 and Hsp20 gene families were found to have more than one copy. This finding suggested that these families underwent an expansion, probably due to gene duplication events, a phenomenon reported to be frequent in *I. scapularis* and other tick species (Gulia-Nuss et al. 2016; Van Zee et al. 2016). To test this hypothesis, we performed a maximum likelihood (ML) phylogenetic analysis using Hsp20 and Hsp70 amino acid sequences. The ML reconstruction of the evolution of *I. scapularis* Hsp20 family members revealed that four (sHspB6B, C, D and E) and two (sHspB5A and D) of the sHspB6 and sHspB5 gene copies are closely related Fig. 15.1a. Similarly, the ML phylogenetic tree using Hsp70 family members shows that two copies of Hsp70–2 (A and C) and HspA9 (A and B) are closely related Fig. 15.1b. These proteins were found to share high percentage identity (sHspB6B, C, D and E, 84% to 96%; sHspB5A and D, 45%; Hsp70–2A and C, 96%; HspA9A and B, 62%). Taken together, these results suggest that gene duplications may have played an important role in *I. scapularis* Hsp70 and Hsp20 evolution. Interestingly, despite being present in the genome, the mRNA for

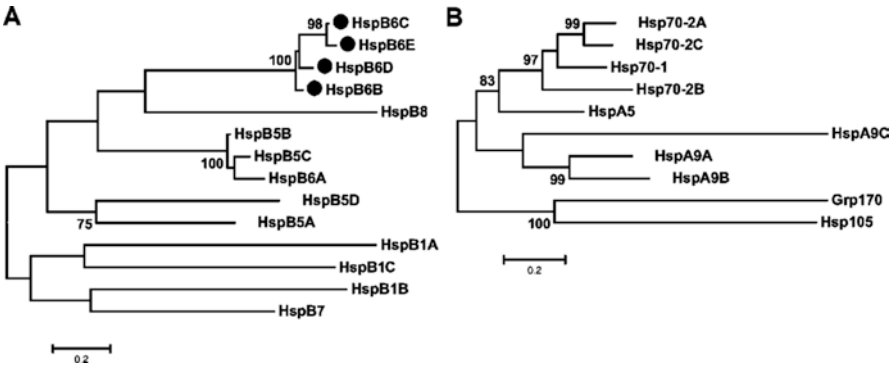

Fig. 15.1 Phylogenetic tree of *I. scapularis* Hsp20 and Hsp70. Unrooted maximum likelihood phylogenetic trees were constructed using *I. scapularis* Hsp20 (**a**) and Hsp70 (**b**) family members. Putative gene duplications of HspB6, HspB5, Hsp70–2 and HspA9 are labeled as black, red, blue and green circles, respectively. Only bootstrap values higher than 70% are shown. Name of Hsp were abbreviated as in Table 15.1. Methods: *I. scapularis* Hsp sequences were collected and aligned using MAFFT (Katoh and Standley 2013). The best-fit model of the sequence evolution was selected based on Corrected Akaike Information Criterion (cAIC) and Bayesian Information Criterion (BIC) implemented in Molecular Evolutionary Genetics Analysis (MEGA) 6 (Tamura et al. 2013). Maximum likelihood (ML) method, implemented in MEGA 6, was used to obtain the best tree topology. Reliability of internal branches was assessed using the bootstrapping method (1000 replicates)

some of these gene copies (labeled with two asterisks in Table 15.1) were not found in *I. scapularis* nymph, adult or ISE6 cell transcriptomes and proteomes, raising the possibility that they may be pseudogenes. This putative pseudogenization in Hsp70 and Hsp20 families would represent a hallmark of the birth-and-death process after duplication events in *I. scapularis* Hsp evolution (Nei and Rooney 2005; Krenek et al. 2013).

15.1.2 Role of HSP in Vector-Pathogen Interactions

Hsp genes are important markers of stress and appear to function as proteins conferring protection to increase tick survival and vector competence in different arthropods (Table 15.2). The expression of different Hsp genes is induced and modulated in insects in response to environmental changes including abiotic stresses such as heat shock and biotic stimulus such as infection by bacteria, viruses and fungi (Zhao and Jones 2012). Arthropod vectors such *Aedes albopictus* and *Aedes aegypti* mosquitoes are known to differentially express Hsp in response to thermal stress (Gross et al. 2009; Sivan et al. 2017). Proteome studies of *Anopheles gambiae*, an important malaria vector, have shown up-regulation of the molecular chaperone Hsp20 in the mosquito head during *Plasmodium* infection (Lefevre et al. 2007), suggesting an

Table 15.2 Examples of Hsps gene expression in ticks and other arthropod vectors during vector-pathogen interactions

Species	Hsp	Effect	Reference
Ixodes scapularis tick cells	Hsp70 Hsp90 Hsp60	Up-regulated Up-regulated Up-regulated	Villar et al., (2015a)
	Hsp20	Down-regulated	Villar et al. (2010)
Ixodes scapularis tick tissues	Hsp20 Hsp70	Up-regulated Down-regulated	Busby et al. (2012)
Anopheles gambiae	Hsp20	Up-regulated	Lefevre et al. (2007)
Anopheles albimanus	Hsp70	Down-regulated	Alvarado-Delgado et al. (2016)
Aedes albopictus	Hsp70	Up-regulated	Tatem and Stollar, (1989)

alteration of the proteome that could also induce behavioral modifications. Hsp70 appears to be also down-regulated in the brain of *Anopheles albimanus* during *Plasmodium* invasion (Alvarado-Delgado et al. 2016). It has been reported that stress proteins such as Hsp70 are induced in *Aedes albopictus* cells after infection with viruses such as Sindbis virus (Tatem and Stollar 1989). However, experiments with Mayaro virus in an *A. albopictus* cell line indicated that the virus is not able to reprogram gene expression induced by heat shock (da Costa Carvalho and Fournier 1991).

At the tick-pathogen interface, pathogens induce several responses including the induction of Hsp to heighten tick survival and favor pathogen infection and transmission (de la Fuente et al. 2016a). Proteomics analysis of *I. scapularis* ISE6 tick cells in response to *A. phagocytophilum* infection showed that some Hsp such as the Hsp70 family were over-represented while other Hsp such as Hsp20 were under-represented in response to infection Fig. 15.2 (Villar et al. 2010). Analysis of *A. phagocytophilum* proteins differentially represented during infection in ticks revealed that Hsp70 is over-represented and interacts with and binds to tick cells, thus playing a role in tick-pathogen interactions (Villar et al. 2015b). Additionally, *A. phagocytophilum* GroEL, also over-represented in high-percentage infected tick cells and salivary glands, may prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions (Villar et al. 2015b). Busby et al. (2012) observed that Hsp20 and Hsp70 mRNA levels were significantly higher and lower, respectively, in *I. scapularis* female ticks fed on sheep infected with *A. phagocytophilum*. Furthermore, the expression of Hsp20 in response to pathogen infection was higher in guts than in salivary glands, while the expression of Hsp70 presented the opposite profile (Busby et al. 2012). Infection and transcriptomics analysis of the *Ixodes ricinus* tick cell line IRE/CTVM20 infected with the viruses LIV (Louping-ill virus) and TBEV (Tick-borne encephalitis virus), and the bacterium *A. phagocytophilum*, identified significant up-regulation of the gene encoding for Hsp70 induced by the three pathogens, which may suggest its potential involvement in inhibition of apoptosis (Mansfield et al. 2017). Villar et al. (2015a) observed that Hsp70 and Hsp90 were over-represented in *A. phagocytophilum*-infected ISE6 tick cells when compared to uninfected cells,

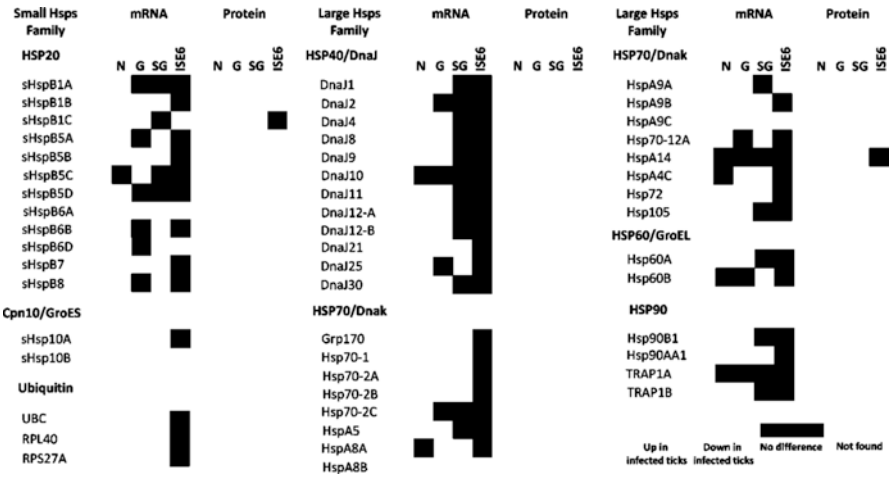

Fig. 15.2 mRNA and protein levels of *I. scapularis* Hsp in response to *A. phagocytophilum* infection. Comparison of Hsp mRNA and protein levels in *I. scapularis* nymphs (N), female midguts (G), female salivary glands (SG) and ISE6 cells (ISE6) in response to *A. phagocytophilum* infection. Transcriptomics and proteomics data were obtained from previously published datasets available on the Dryad repository database, NCBI's Gene Expression Omnibus database and ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002181 and doi: https://doi.org/10.6019/PXD002181 (Ayllón et al. 2015; Villar et al. 2015a). Name of Hsp were abbreviated as in Table 15.1. The datasets used in this analysis on the tick transcriptomics and proteomics response to *A. phagocytophilum* infection have been validated before in several studies (Ayllón et al. 2015; Villar et al. 2015a; Cabezas-Cruz et al. 2016, 2017a, b)

suggesting a role for these proteins in counteracting the negative effect of heat shock and pathogen infection on tick questing behavior and survival.

15.1.3 *Role of HSP in the Interactions Between* A. phagocytophilum *and* I. scapularis *Tick Vector*

The Hsp response to *A. phagocytophilum* infection was characterized using the quantitative transcriptomics and proteomics data generated from uninfected and *A. phagocytophilum*-infected *I. scapularis* ticks and ISE6 cultured cells, which represent a model for tick hemocytes (Ayllón et al. 2015; Villar et al. 2015a). As in previous reports for other proteins involved in apoptosis, metabolism, remodeling of the cytoskeleton and epigenetics (Ayllón et al. 2015; Villar et al. 2015a; Cabezas-Cruz et al. 2016, 2017a, 2017b), most of the identified Hsp genes were differentially regulated in response to *A. phagocytophilum* infection in at least one of the analyzed tick samples Fig. 15.2. Nineteen (37.2%), 13 (25.4%), 32 (62.7%), and 32 (62.7%) Hsp were identified in both transcriptome and proteome of ISE6 cells, nymphs, adult female midguts and salivary glands, respectively Fig. 15.2. The proteomics

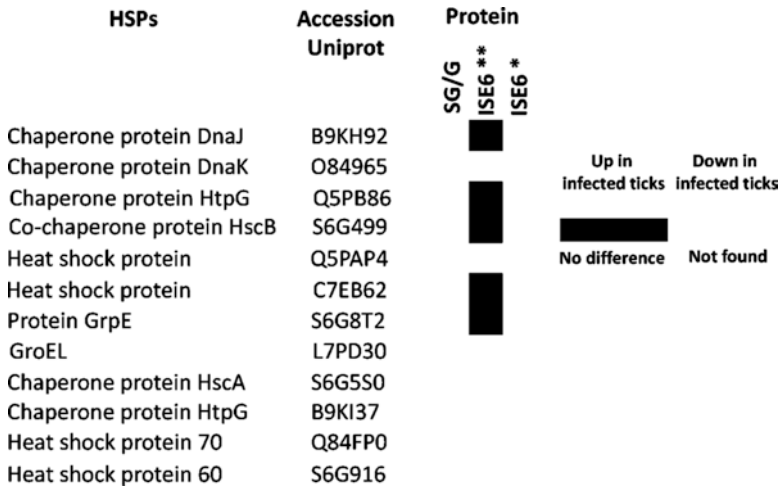

Fig. 15.3 Protein levels of *A. phagocytophilum* Hsp in ticks and ISE6 cells. Comparison of *A. phagocytophilum* Hsp levels in salivary glands versus midgut (SG/G), infected versus uninfected ISE6 cells (ISE6*) and early (3 days post-infection) versus late (8 days post-infection) infection in ISE6 cells (ISE6**). Data collected from Ayllón et al. (2015), Villar et al. (2015b), and Gulia-Nuss et al. (2016)

results showed that various proteins were not identified in one or several samples Fig. 15.2, suggesting low protein levels in these cells or tissues. However, the levels of different proteins significantly changed in response to infection Fig. 15.2. Considering the protein levels to provide an indicator of the effect of *A. phagocytophilum* infection on tick Hsp, the results showed that 64% of all identified Hsp were over-represented, suggesting a global increase in response to infection. These results supported the presence of tissue-specific differences in the tick cell response to infection (Ayllón et al. 2015; Villar et al. 2015a; Cabezas-Cruz et al. 2016, 2017a, 2017b).

To evaluate the response of *A. phagocytophilum* to infection in ticks, we focused on proteomics data for midguts, salivary glands and ISE6 cells (Ayllón et al. 2015; Villar et al. 2015a, 2015b; Gulia-Nuss et al. 2016). Except for one bacterial Hsp (accession number C7EB62), all identified *A. phagocytophilum* Hsp were over-represented in tick salivary glands in response to infection when compared to midguts Fig. 15.3. A similar response was observed in infected versus uninfected ISE6 cells. However, only two Hsp were over-represented when early versus late infection were compared. These data suggest that infection in ticks triggers the stress response in *A. phagocytophilum*. However, once the infection is established, the bacterium does not require further regulation of Hsp levels. Immunofluorescence in adult female ticks was performed to validate proteomics data using anti-Hsp70 and anti-Hsp90 antibodies Fig. 15.4. The results showed that the immunofluorescence (arrows in Fig. 15.4) was mainly associated with salivary glands using labeled anti-Hsp70 and anti-Hsp90 antibodies in uninfected and *A. phagocytophilum*-infected

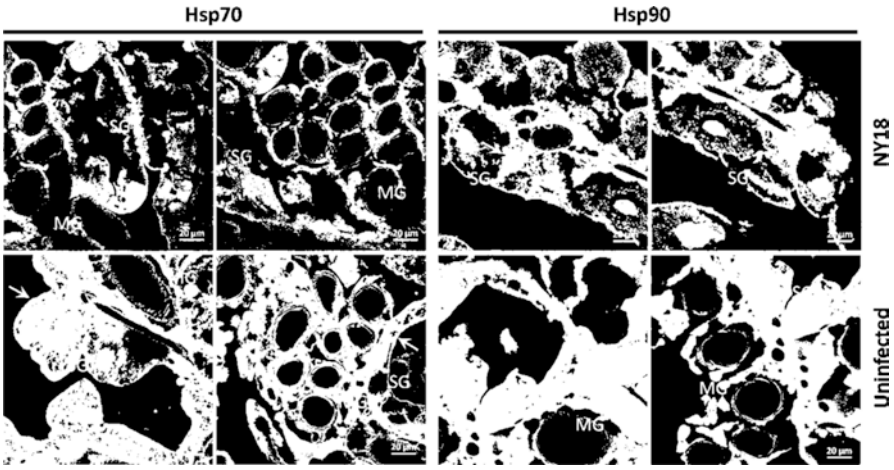

Fig. 15.4 Immunofluorescence in adult female ticks. Representative images of immunofluorescence analysis of uninfected and *A. phagocytophilum*-infected adult female *I. scapularis* ticks. Tick tissues were stained with anti-Hsp70 or anti-Hsp90 labelled with FITC (*green*) and DAPI (*blue*). The immunofluorescence (*arrows*) was mainly associated with salivary glands labelled with anti-Hsp70 and anti-Hsp90 in uninfected and *A. phagocytophilum*-infected ticks, respectively. No clear immunofluorescence was associated to infected or uninfected midguts. Experimental procedures: *I. scapularis* ticks were obtained from the laboratory colony maintained at the Oklahoma State University Tick Rearing Facility. Nymphs and adult female *I. scapularis* were infected with *A. phagocytophilum* by feeding on a sheep inoculated intravenously with approximately $1x10^7$ *A. phagocytophilum* (NY18 isolate)-infected HL-60 human cells (90–100% infected cells) (Kocan et al. 2012; Ayllón et al. 2015). Female ticks were removed from the sheep 10 days after infestation, held in the humidity chamber for 4 days and fixed with 4% paraformaldehyde in 0.2 M sodium cacodylate buffer, dehydrated in a graded series of ethanol and embedded in paraffin. Sections (4 µm) were prepared and mounted on glass slides. The paraffin was removed from the sections with xylene and the sections were hydrated by successive 2 min washes with a graded series of 100, 95, 80, 75 and 50% ethanol. The slides were treated with Proteinase K (Dako, Barcelona, Spain) for 7 min, washed with PBS and incubated with 3% bovine serum albumin (BSA; Sigma-Aldrich) in PBS for 1 h at room temperature. The slides were then incubated for 14 h at 4 °C with primary antibodies anti-Hsp70 and Hsp90 (Sigma-Aldrich) diluted 1:100 in 3% BSA/PBS and, after 3 washes in PBS, developed for 1 h with goat-anti-mouse IgG conjugated with FITC (Sigma-Aldrich) (diluted 1:200 in 3% BSA/PBS). The slides were washed twice with PBS and mounted in ProLong Antifade with DAPI reagent (Molecular Probes, Eugene, OR, USA). The sections were examined using Zeiss LSM 800 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). Sections of uninfected ticks and IgGs from preimmune serum were used as controls

ticks, respectively. No clear immunofluorescence was associated to infected or uninfected midguts. This result clearly validates the proteomics data, which showed that the tick Hsp90 (Hsp90B1 and Hsp90AA1) identified in this study was over-represented in infected salivary glands Fig. 15.2. However, the low fluorescence signal in infected salivary glands using the labeled anti-Hsp70 antibodies could be due to poor recognition of tick protein by these antibodies. Therefore, this result did not allow making conclusions for Hsp70–1, Hsp70–2B and Hsp70–2C proteins that were also over-represented in the proteome of infected salivary glands Fig. 15.2.

15.1.4 Anti-Apoptotic Function HSP

The inhibition of cell apoptosis is a common mechanism used by intracellular bacteria to facilitate infection (de la Fuente et al. 2017). In ticks, *A. phagocytophilum* infection results in the inhibition of apoptosis to facilitate infection while tick cells respond by activating alternative apoptosis pathways to control infection and preserve tick fitness (Ayllón et al. 2015; de la Fuente et al. 2017). There is consensus in that both large and sHsp have anti-apoptotic function (Takayama et al. 2003). Several sHsp such as sHspB1, sHspB2 and sHspB5 are involved in apoptosis inhibition in mammals (Bakthisaran et al. 2015). In particular, sHspB1 (Hsp27) leads to delayed release of cytochrome c from mitochondria, thus interfering with intrinsic apoptosis pathway, sHspB5 (αB-crystallin) prevents cell death induced upon oxidative stress, and sHspB1 (Hsp27) and sHspB2 (MKBP) inhibit the extrinsic apoptosis pathway (Bakthisaran et al. 2015). At least one of these mechanisms is used by *A. phagocytophilum* to inhibit apoptosis in tick salivary glands through the inhibition of cytochrome c release by down-regulation of tick Porin protein levels (Ayllón et al. 2015). In addition to Porin, our results suggested that tick sHsp might be also involved in apoptosis inhibition by *A. phagocytophilum* infection in midguts and salivary glands. All copies of sHspB1 and sHspB5 found in the *I. scapularis* genome were over-represented in infected midguts and at least three of them (sHspB1B, sHspB5B and C) also in infected salivary glands Fig. 15.2. To provide additional support for the role of these Hsp during pathogen infection, the expression of sHspB1 and sHspB5 was silenced by RNA interference in ISE6 tick cells. The silencing of sHspB1 decreased *A. phagocytophilum* levels within tick cells and increased tick cell apoptosis Fig. 15.5. These results showed that sHspB1 has an anti-apoptotic effect in tick cells that is beneficial for *A. phagocytophilum* infection of tick cells. Another Hsp involved in apoptosis inhibition is Hsp70 (Beere et al. 2000). Hsp70 inhibits apoptosis by numerous pathways (Mosser et al. 1997; Beere et al. 2000; Ravagnan et al. 2001; Shukla et al 2003; Shukla et al. 2014). One of

Fig. 15.5 (continued) CTCTTCGACGACGACTTCGGC and reverse CGGCGCGAAGTGAC GGACGTC) and the Kapa SYBR Fast One-Step qRT-PCR Kit (Kapa Biosystems) and the QIAGEN Rotor-Gene Real-Time PCR Detection System. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently in the same temperature range for every sample. The mRNA levels were normalized against tick *rpS4* using the genNorm method (Delta-Delta-Ct, ddCT) as described previously (Ayllón et al. 2015). Normalized Ct values were compared between test dsRNA-treated tick cells and controls treated with *Rs86* dsRNA, and between infected and uninfected tick cells by Student's t-test with unequal variance (P=0.05; N=4 biological replicates). The percentage of apoptotic cells was measured as previously described (Ayllón et al. 2015). Briefly, apoptosis was measured by flow cytometry (FACS) using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Immunostep, Salamanca, Spain). Cells were stained simultaneously with the non-vital dye propidium iodide (PI) allowing the discrimination of intact cells (Annexin V-FITC negative, PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative), late apoptotic/necrotic cells (Annexin V-FITC positive, PI positive) and dead cells (Annexin V-FITC negative, PI positive) The percentage of apoptotic cells (including early apoptotic, late apoptotic/necrotic and dead cells) was determined by FACS after Annexin V-FITC and PI labeling

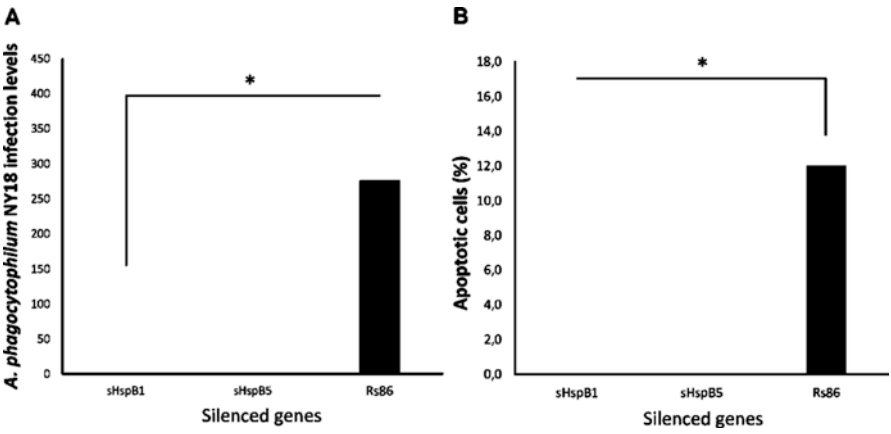

Fig. 15.5 RNA interference for sHsp gene knockdown in tick cells. The *A. phagocytophilum* DNA levels were determined after RNA interference in infected ISE6 tick cells treated with sHspB1 and sHspB5 dsRNAs or control Rs86 dsRNA. *A. phagocytophilum* DNA levels were determined by *msp4* real-time PCR normalizing against tick *rpS4*. Results are shown as average + S.D. normalized Ct values and compared between treated and control groups by Student's t-test with unequal variance ($P<0.05$; N=4 biological replicates) (**a**). The percentage of apoptotic cells was determined by flow cytometry in ISE6 tick cells treated with sHspB1 and sHspB5 dsRNAs or control Rs86 dsRNA (**b**). Experimental procedures for RNA interference to characterize the effect of gene knockdown on tick cell pathogen infection and gene expression: Oligonucleotide primers targeting *I. scapularis* sHspB1C (ISCW023475: forward CGACGACTTCTTCGACTTCC and reverse CGCTCATAGATGCTGCTTGT) and sHspB5 (B and C) (ISCW002513 and ISCW024922: forward CTCTTCGACGACGACTTCGGC and reverse GCCTCCACGGCCAGCAGTCCT) and containing T7 promoters were used for *in vitro* transcription and synthesis of dsRNA as described previously (Ayllón et al. 2013), using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA). The unrelated *Rs86* dsRNA was synthesized using the same methods described previously and used as negative control (Ayllón et al. 2013). The dsRNA was purified and quantified by spectrophotometry. RNAi experiments were conducted in cell cultures by incubating ISE6 tick cells with 10 µl dsRNA ($5x10^{10}$-$5x10^{11}$ molecules/µl) and 90 µl L15B300 medium in 24-well plates using 4 wells per treatment. Control cells were incubated with the unrelated *Rs86* dsRNA. After 48 hours of dsRNA exposure, tick cells were infected with cell-free *A. phagocytophilum* NY18 obtained from approximately $5x10^6$ infected HL-60 cells (90–100% infected cells) (Thomas and Fikrig 2007) and resuspended in culture medium to use 1 ml/well or mock infected by adding the same volume of culture medium alone. Cells were incubated for an additional 72 hours, harvested and used for DNA and RNA extraction. RNA was used to analyze gene knockdown by real-time RT-PCR with respect to *Rs86* control. DNA was used to quantify the *A. phagocytophilum* infection levels by major surface protein 4 gene (*msp4*) PCR. Determination of *A. phagocytophilum* infection by real-time PCR: *A. phagocytophilum* DNA levels were characterized by *msp4* real-time PCR normalizing against tick *ribosomal protein S4* (*rpS4*) as described previously (Ayllón et al. 2015). Normalized Ct values were compared between untreated and treated cells by Student's t-test with unequal variance ($P=0.05$; N=4 biological replicates). Determination of mRNA levels by real-time RT-PCR: total RNA was extracted from the same cell cultures (N=4) using TriReagent (Sigma, St. Louis, MO, USA) following manufacturer's recommendations. The expression of sHsp genes was characterized using total RNA extracted from infected and uninfected ISE6 tick cells. Real-time RT-PCR was performed on RNA samples using gene-specific oligonucleotide primers targeting sHspB1C (ISCW023475: forward CGACGACTTCTTCGACTTCC and reverse GTGTTCGTCGTGTGGTGAAC) and sHspB5 (B and C) (ISCW002513 and ISCW024922: forward

these mechanisms involves the prevention of cytochrome c/dATP-mediated caspase activation (Beere et al. 2000). Our results showed that several members of Hsp70 families are over-represented in *A. phagocytophilum* infected ticks and ISE6 cells Fig. 15.2 (Villar et al. 2015a).

15.1.5 HSP and Vector Tolerance to Pathogen Infection

In response to pathogen infection, tick innate immune response pathways are activated to control pathogen infection and multiplication (Hajdušek et al. 2013; Mansfield et al. 2017; Shaw et al. 2017; de la Fuente et al. 2017). It is generally assumed that the main function of the vector innate immune system in response to pathogen infection is pathogen clearance. However, there is tight relationship between immunity to pathogen infection and vector competence. Strong immune response to pathogen infection is associated with a dramatic decreased in vector competence (Johns et al. 2001). For example, while *I. scapularis*, also the vector of *Borrelia burgdorferi* is immunotolerant to spiroquete infection, *Dermacentor variabilis*, which is unable to transmit *B. burgdorferi*, shows a very potent immune response against this pathogen (Johns et al. 2001). The molecular bases of vector tolerance to pathogen infection are far from being understood. However, recent studies showed that tick vector competence is also affected by the ability of transmitted pathogens to evade tick innate immune response (Hajdušek et al. 2013; Shaw et al. 2017; de la Fuente et al. 2017), which highlights the capacity of pathogens to trigger immunotolerance in tick vectors. Interestingly, Hsp are among the molecular factors activated in response to *A. phagocytophilum* infection (Villar et al. 2010; Villar et al. 2015a), and in addition to the anti-apoptotic function described above, Hsp are also considered to be directly liked to suppression of the immune system in vertebrate animal models (van Noort et al. 2012). In particular, sHsp such as sHspB1, sHspB4 and sHspB5 were found to modulate the activity of phagocytic cells such as macrophages (De et al. 2000; van Noort et al. 2010). A recent study has shown that sHspB1 causes differentiation of monocytes to macrophages with tolerizing phenotypes involving low expression of MHC class II molecules and the costimulatory surface protein CD86, and high expression of several co-inhibitory surface molecules (Banerjee et al. 2011). This tolerizing macrophage phenotype suppresses cellular immune reactions (Banerjee et al. 2011). Another study showed that sHspB1 and sHspB5 induce macrophages to express tolerizing cytoquines such IL-10 (De et al. 2000; van Noort et al. 2010). sHspB5 also exhibits anti-inflammatory effects by temperature-dependent binding of pro-inflammatory proteins in plasma, which can in turn influence both the innate and the adaptive immune responses (Bakthisaran et al. 2015).

Ticks have immune cells with macrophage-like functions that are called hemocytes (Hajdušek et al. 2013). At least three types of hemocytes have been recognized in the hard and soft ticks, of which two, plasmatocytes and granulocytes I, are phagocytic (Sonenshine 1991; Borovickova and Hypsa 2005). Tick hemocytes have the capacity to engulf foreign material and different microbes (Inoue et al. 2001; Loosova et al. 2001; Buresova et al. 2006). However, little is known about immune tolerance in arthropod vectors (Baxter et al. 2017).

Interestingly, we identified a sHsp family in *I. scapularis* with evidence of significant expansion in the number of sHspB1 and sHspB5 paralogs due, apparently, to gene duplication Fig. 15.1 and Table 15.1. The response of tick sHspB1 (A and B) and sHspB5 (A, B, C and D) to *A. phagocytophilum* infection was characterized by an over-representation of these proteins in infected tissues from adult ticks Fig. 15.2. Only four of these proteins sHspB1 (A and C) and sHspB5 (A and D) were found to be under-represented in midguts (sHspB1C) and salivary glands (sHspB1A and sHspB5A and D). At least for sHspB1 and sHspB5 the results do support a role for sHsp in tick tolerance to *A. phagocytophilum* infection. Indeed, sHspB1A is also over-represented in *A. phagocytophilum*-infected ISE6 cells Fig. 15.2, which are a model of tick hemocytes (Villar et al. 2015a).

A recent study showed that maintaining glucose levels is a key metabolic adaptation to establish disease tolerance to sepsis in a mice model (Weis et al. 2017). We previously showed that *A. phagocytophilum* decreases the levels of glucose in infected cells (Villar et al. 2015a), but at the same time the glucose transporter GLUT1A is over-represented in salivary glands and midguts of *I. scapularis* infected by *A. phagocytophilum* (Cabezas-Cruz et al. 2017a). GLUT1A is under the control of the transcription factor hypoxia inducible factor 1 alpha (HIF-1α), which is stabilized by Hsp70 and Hsp90 in normoxia (Zhou et al. 2004). As previously reported (Villar et al. 2015a) and shown in Fig. 15.2, Hsp70 and Hsp90 are over-represented in *A. phagocytophilum*-infected salivary glands and ISE6 tick cells when compared to uninfected controls. Altogether, these results suggest that by stabilizing HIF-1α, tick Hsp70 and Hsp90 may induce the expression of GLUT1A to increase the glucose uptake and infection tolerance in response to *A. phagocytophilum* infection.

15.1.6 Possible Applications of HSP as Vaccine Antigens for the Control of Tick-Borne Diseases

Hsp may represent attractive potential vaccine candidates to be used alone or in synergy with other antigens for the control of tick infestations and pathogen infection and transmission with the ultimate goal of reducing the burden of tick-borne diseases. They possess antigenic peptides that mediate the maturation and stimulation of dendritic cells, which are necessary for the secretion of inflammatory cytokines (Newport 1991; Suto and Srivastava 1995; Zugel and Kaufmann 1999).

A sHsp from *Rhipicephalus annulatus* ticks (Ra-sHSPI) appears to be highly immunogenic in rabbits, suggesting that it could be used as a potential protective antigen (Hussein et al. 2014). The screening of antigenic proteins derived from *Dermacentor silvarum* identified Hsp60 (a heat shock protein and a chaperonin) as a highly immunogenic protein with the potential to be used to prevent both tick infestation and pathogen transmission by *D. silvarum* (Zhang et al. 2015). Studies on the identification of *I. scapularis* salivary immunogenic proteins during the first 24 hours of feeding found, amongst others, the Hsp90 (Lewis et al. 2015). Hsp90 could be a candidate protective antigen against bacterial infection (Pockley 2003). This approach can provide good candidates to affect tick feeding before pathogen transmission occurs (Lewis et al. 2015). However, immunization of rabbits with the

recombinant *Haemaphysalis longicornis* Hsp70 did not result in a statistically significant reduction of female tick engorgement and oviposition (Tian et al. 2011). This result suggests that, although HLHsp70 plays a role in many cellular processes, it is not a candidate vaccine antigen against *Haemaphysalis* tick infestations (Tian et al. 2011). Immunization of mice using recombinant *Babesia gibsoni* and *Babesia microti* Hsp70 induced high antibody levels and significant reductions in peripheral parasitaemias (Terkawi et al. 2009). Studies with *Babesia bovis* Hsp20 indicated that this protein was recognized by CD4 T lymphocytes from cattle that have recovered from infection and were immune to tick challenge (Norimine et al. 2004), suggesting that it could be used to design multiepitope vaccines. Li et al. (2005) observed that fusion antigen P1-HspB was a good immunogen for eliciting an immune response against *Coxiella burnetii*, and could be a more suitable candidate for preparing subunit vaccines against Q fever than P1 (a cell surface component) or HspB (a member of the Hsp60 family) alone. Hsp-20 from *B. bigemina* appears to be expressed in both sexual stages and kinetes throughout the life cycle of the parasite, suggesting that it plays an important role in the cellular physiology of tick stages and could be a potential vaccine candidate (Vichido et al. 2008). A high throughput quantitative proteomics approach was used to characterize *A. phagocytophilum* proteome response during bacterial multiplication, and to identify proteins involved in infection of the tick vector, *I. scapularis* (Villar et al. 2015b). In this study, bacterial Hsp70 was over-represented in infected tick cells and salivary glands when compared to low percentage infected cells and midguts, suggesting that it plays a role in rickettsia-tick interactions. Therefore, *A. phagocytophilum* Hsp70 could be used as a candidate protective antigen in vaccines for the control of *Anaplasma* infection in tick vectors by reducing or blocking transmission to vertebrate hosts.

Other proteins that could be used for the development of vaccines for the control of *A. phagocytophilum* infection in ticks by reducing or blocking transmission to the vertebrate host are major surface proteins 4 (MSP4) and GroEL (Villar et al. 2015b). A recent study by Contreras et al. (2017) showed that MSP4 and Hsp70 are involved in host-pathogen interactions. Although they were only partially protective against pathogen infection in sheep, they could be combined with other candidate protective antigens for the development of vaccines for the control of human and animal granulocytic anaplasmosis. Additionally, it has been shown that *Ehrlichia muris* GroEL can stimulate the production of IFNγ in CD4 T cells, but not in naïve T cells, and could also induce B cells and T cells therefore suggesting that it could be a good vaccine candidate to protect against *E. muris* infection (Thomas 2016).

15.2 Conclusions

This review focused on the role of *I. scapularis* and *A. phagocytophilum* Hsp during tick-pathogen interactions. The results show that the transcript and protein levels of tick and bacteria Hsp are regulated in response to *A. phagocytophilum* infection in the tick vector *I. scapularis*. Furthermore, *A. phagocytophilum* GroEL and Hsp70 are involved in pathogen binding to tick cells and are secreted by the bacterium once

it reaches the cytoplasm of infected tick cells, presumably through T4 bacterial secretion system (T4SS). Further studies should clarify whether *A. phagocytophilum* Hsp70 can inhibit tick cell apoptosis. As reported for other host genes (Garcia-Garcia et al. 2009a, b; Rennoll-Bankert et al. 2015), the transcriptional regulation of tick Hsp by *A. phagocytophilum* infection can be linked to epigenetic modifying functions of effector proteins secreted by the bacteria. Here we showed that tick sHspB1 is over-represented in tick ISE6 cells Fig. 15.2, with an anti-apoptotic function and involved in *A. phagocytophilum* survival within ticks cells Fig. 15.5. We propose a mechanism to explain the contribution of Hsp to tick apoptosis inhibition in response to *A. phagocytophilum* infection Fig. 15.6. The present work provides a

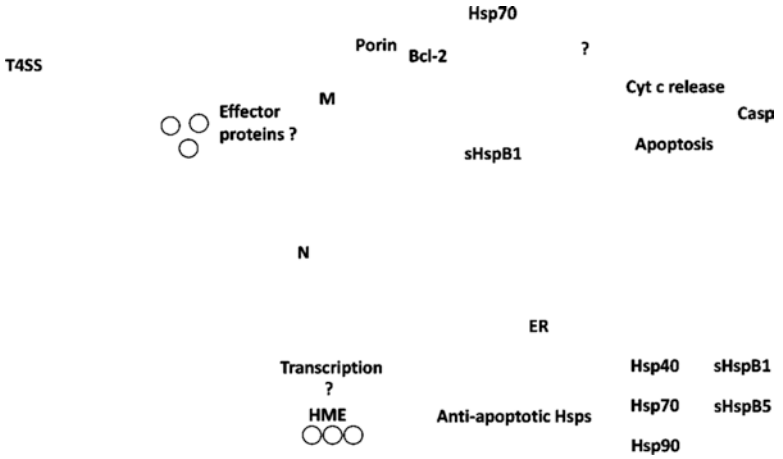

Fig. 15.6 Proposed mechanism of the contribution of Hsp to tick apoptosis inhibition in response to *A. phagocytophilum* infection. Apoptosis is major cell defense mechanism against intracellular pathogens. Pathogens inhibit vector cell apoptosis using different mechanisms. After infection of tick salivary glands, *A. phagocytophilum* inhibit apoptosis by decreasing the expression of the pro-apoptotic genes coding for proteins such as ASK1 and Porin. Porin down-regulation is associated with the inhibition of mitochondrial Cyt c release (Ayllón et al. 2015). In contrast, *A. phagocytophilum* infection does not affect Bcl-2 levels, probably because this protein but not Porin is essential for tick feeding (Ayllón et al. 2015). The capacity of *A. phagocytophilum* to regulate gene expression in neutrophils was associated with histone modifying enzymes recruitment to the promoters of target genes by effectors proteins such as the ankyrin repeat protein AnkA (Garcia-Garcia et al. 2009a, b; Rennoll-Bankert et al. 2015). *A. phagocytophilum* Hsp70 and GroEL proteins were overrepresented in infected ticks or cells and can interact and bind to tick cells (Villar et al. 2015b). The *A. phagocytophilum* T4SS system may be associated with the secretion of Hsp70 and other stress response proteins (Villar et al. 2015b). Abbreviations: nucleus (N), mitochondria (M), endoplasmic reticulum (ER), Cytochrome c (Cyt c), caspases (Casp) and histone modifying enzymes (HME). The molecules represented as green ovals with black (*I. scapularis*) and white (*A. phagocytophilum*) letters are proteins up-regulated in response to *A. phagocytophilum* infection. Molecules represented as white ovals are components of the apoptosis pathway but are no Hsp. The *A. phagocytophilum* MSP4 binds tick cells and is also shown, but it is not a HSP (Villar et al. 2015b)

comprehensive view of the Hsp involved in the response to pathogen infection in ticks, and provides the basis for further studies to develop novel strategies for the control of human granulocytic anaplasmosis.

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