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Use of *Artemia* as Model Organism to Study Epigenetic Control of Phenotypes Relevant for Aquaculture Species

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LABORATORY OF AQUACULTURE & ARTEMIA REFERENCE CENTER

That which does not kill us makes us stronger

- Friedrich Nietzsche (1844 -1900)

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DEDICATION

This thesis is dedicated to my beloved husband **Dr. Kartik Baruah** who made the difference in my life and never stopped supporting and encouraging me.

This thesis is dedicated to my loving parents, **Pari Abdollahi Moghaddam** and **Hammid Reza Norouzitallab** whose words of encouragement and push for tenacity helped me through the entire process. To my little brother **Mehrab,** who never left my side and he is very special to me.

I dedicate this work and give special thanks to my beloved Family in Belgium **Denise De Nul** and **Marc D´hont** for being there for me during every steps of my life in Belgium.

This thesis work is dedicated to my beloved **Dr. Ana Bossier**, for her constant support and encouragement.

Contents

List of Abbreviations

°C	Degree Celsius
%	Percentage
±	Approximately
/	Per
µmol	Micromoles
µg	Microgram
µg	Microgram
C	Control
CCAP	Culture Collection of Algae and Protozoa
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
dG	deoxyguanosine
dscam	Down syndrome cell adhesion molecule - mRNA
ftn	*Ferritin* - mRNA
g/L	gram per liter
g	Gram
g	Relative centrifugal force or G force
h	Hour
H	Histone
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HMGB1	High mobility group box 1 - protein
hmgb1	High mobility group box 1- mRNA
HSF	Heat shock factor
HSP70	Heat shock protein 70 - protein
hsp70	Heat shock protein 70 - mRNA
HSP	Heat shock protein
HSPs	Heat shock proteins
IgG	Immunoglobulin G
L	Liter
lgbp	Beta-1,3-glucan-binding protein - mRNA
M^2	Meter square
min	Minute
mdC	5-methyl-2-deoxycytidine
$MgCl_2$	Magnesium chloride
mL	Milliliter
mm	Millimeter
mM	Millimoles
mRNA	Messenger ribonucleic acid
m/z	Mass to charge ratio
n	Number of replicates
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate monohydrate
nm	Nano meter
ng	Nano gram
P	Statisctical P value obtained
PCR	Polymerase chain reaction
pH	Measure of the acidity of a solution
propo	Prophenoloxidase - mRNA

RT-PCR (qPCR)	Real time Polymerase chain reaction
PVDF	polyvinylidene fluoride membranes
pxn	Peroxinectin - mRNA
rpm	Rotation per minute
RNA	Ribonucleic acid
s	Second
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T	Treatment
tgase	Transglutaminase - mRNA
UHPLC	Ultra-High Performance Liquid Chromatography
VC	*Vibrio campbellii*

PART I

Introduction and Literature Review

Imagination is more important than knowledge

- Albert Einstein (1879 – 1955)

CHAPTER 1

Thesis Outline and Objectives

Global fish production and food security – an overview

Food security is of fundamental importance for a healthy human population. Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary requirements and food preferences for an active and healthy life (Drewnowski, 2010).

Seafood and in particular fish and shellfish has long been considered as one of the main sources of protein all over the world. Fish and shellfish production from fisheries and aquaculture improved the human population health by playing a significant role in reduction of poverty and hunger. A recent FAO report indicated that fish and shellfish accounted for 17 % of the global population's intake of animal protein and 6.5 % of total consumed protein (FAO, 2014). Thus, fish and shellfish in broad sense contributes to food security and economy through directly being used as a food or produced for trade or exports (FAO, 2014).

At present, about 158 million tons of fish and shellfish are produced globally, of which 136 million tons are used for human consumption (FAO, 2014). However, the contribution of the captured fisheries to the world fish and shellfish production in terms of quantity is about 90 million tons and has leveled off at this figure since the 1990s (**Fig. 1.1**). This stagnant state in fisheries production for the past three decades is because most of the industrial fishing areas are exploited to their maximum potentials. Sustaining seafood supplies from capture fisheries to meet the growing global demand for food and for improving global food security is therefore under question.

Aquaculture sector on the other hand is considered as a successful and rather recent industry which has been increasingly contributing to the global food production (from 5% to 50% in less than 40 years; **Fig 1.1**). World aquaculture food production has more than doubled from 32.4 million tons in 2000 to 66.6 million tons in 2012. In 2012, aquaculture contributed to about 42.2% of all fish consumed as human food **(Fig. 1.1)** and this value is expected to increase to 62% by 2030. In 2012, two-thirds of the total aquaculture production (44.2 million tons) was finfish species grown from inland aquaculture (38.6 million tons) and mariculture (5.6 million tons). The farmed crustaceans accounted for 9.7% (6.4 million tons) of aquaculture production by volume but 22.4% (US$30.9 billion) by value. The molluscs production (15.2 million tons) was more than double that of crustaceans, but its value was only half that of crustaceans (**Fig. 1.2**; **Table 1.1**). With significant contribution of the aquaculture sector to the global fish production (since the 1990s, the capture fisheries production has plateaued), the world's fish production has increased almost eight times since 1950 (**Fig. 1.1**). In fact, with an average growth rate of 3.2% per year in the period between 1961 and 2009, the world fish supply has effectively been growing faster than the world's population (FAO, 2014).

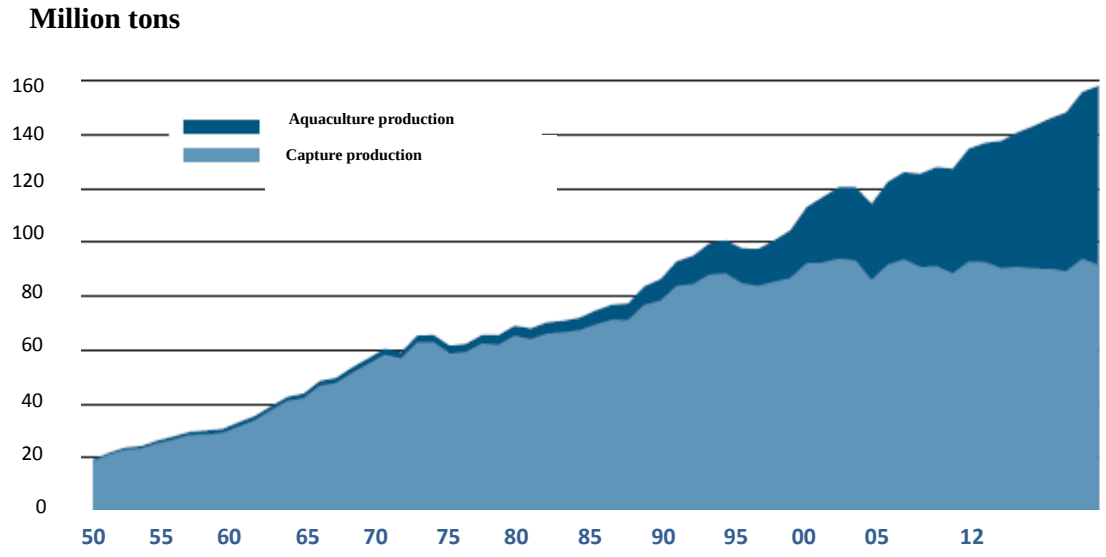

Fig. 1.1: Trend in word fisheries and aquaculture production. While fisheries production growth rate seems to be stagnant since 1190 due to sustainability of the practices, aquaculture experiences a steady increase in growth rate of protein production (FAO, 2014).

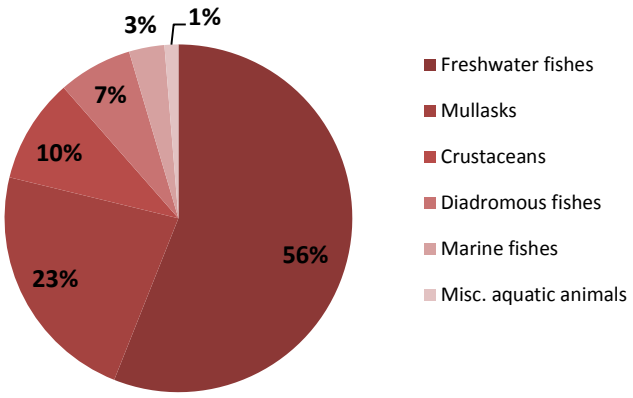

Fig. 1.2: World aquaculture production. Major aquaculture species and their contribution to the total production (adapted from FAO, 2014).

Table 1.1: World production of aquatic species from inland aquaculture and mariculture (adapted from FAO, 2014).

	Inland aquaculture	Mariculture	Quantity subtotal		Value subtotal	
	(Million tons)	*(Million tons)*	*(Million tons)*	*(% by volume)*	*(US$million)*	*(% by value)*
Finfish	38.599	5.56	44.151	66.30	87 499	63.5
Crustacean	2.530	3.917	6.447	9.70	30 864	22.4
Molluscs	0.287	14.89	15.171	22.80	15 857	11.5
Other species	0.530	0.335	0.865	1.30	3 512	2.5
Total	41.95	24.69	66.63	100	137 732	100

Given the above information, aquaculture remains to be an important food producing sector that can make a significant contribution to the increasing demand for safe and quality aquatic food and eventually to global food security. However, despite all the success, the aquaculture practices are mostly relying on empirical approaches which is based on trial and error using sound scientific principles. Even though the empirical approaches were successful so far, with great expectations from this sector and the challenges ahead, development of a more knowledge-based procedures are becoming increasingly indispensable (Sorgeloos, 2013). Therefore, it is imperative to understand the underlying mechanisms of all biological processes responsible for the final production outcome in order to develop a more predictable, reliable, cost-effective and ultimately more sustainable industry (Sorgeloos, 2013).

In this context, one of the major constrains in aquaculture that always drew a lot of attention is disease outbreaks resulting in extreme socio-economic losses. Experimental approaches are required in order to understand the causes and to find permanent solutions for this problem.

Aquaculture disease: a threat to global food security

Aquatic animal's disease outbreaks in various parts of the world have markedly disrupted aquaculture production, seriously compromising food security. Disease occurs at all life stages of fish and shellfish during culture, resulting from the stressful environment conditions that favors the proliferation of pathogenic and opportunistic microorganisms (**Fig. 1.3**). Fish and shrimp are affected by many pathogens including protozoa, bacteria, fungi, and viruses (Lightner and Redman, 1998). Disease outbreak imposes a great limitation for shrimp farming and causes severe economic losses. For example, following very profitable decades in the 1970s and 1980s, shrimp aquaculture experienced a very rough decade in the 1990s with severe disease problems caused by poor management practices and uncontrolled transfers of contaminated broodstock or post larvae (Sorgeloos, 2013). In general, disease outbreak has significant impacts on crustacean farming and is partly responsible for the high market value of the shrimps. Among the infectious diseases of cultured shrimp, certain virus-caused diseases stand out as the most significant. Such examples include white spot syndrome (WSSV), infectious hypodermal and hematopoietic necrosis (IHHNV), yellow head (YHV), hepatopancreatic parvovirus (HPV), taura syndrome virus and many others. Losses in terms of economy due to disease outbreaks have been significant and range from a level of US$ 17.5 million to US$ 3.2 billion (Israngkura and Sae-Hae, 2002; FAO, 2007). Besides viruses, bacteria belonging to the *Vibrio* spp. (such as *V. campbellii*, *V. harveyi, V. parahaemolyticus*, *V. alginolyticus, V. anguillarum, V. vulnificus*, etc.) are by far the most serious, causing massive mortalities of cultured shellfish worldwide (Chen *et al.,* 2000; Austin and Zhang, 2006). These bacteria belong to the family *Vibrionaceae* and are Gram-negative, rod shaped, facultative anaerobes, and in most of the cases motile because of their flagellae,

which is increasing their pathogenicity (**Fig. 1.4;** Thompson et al., 2004). *Vibrio* disease is commonly described as vibriosis or bacterial disease, penaeid bacterial septicaemia, penaeid vibriosis, luminescent vibriosis (**Fig. 1.5**) or red-leg disease (Aguirre-Guzmán et al., 2004).

With the rapid developments in aquaculture, particularly in Asia and South America, vibrios have become recognized as a serious cause of disease (Austin and Zhang, 2006). In many cases, vibrios are opportunists, only causing disease when the host organism is immune-suppressed or otherwise physiologically stressed, with the frequency of infection often being attributable to intensive culture and adverse environmental abiotic factors (Peddie and Wardle, 2005).

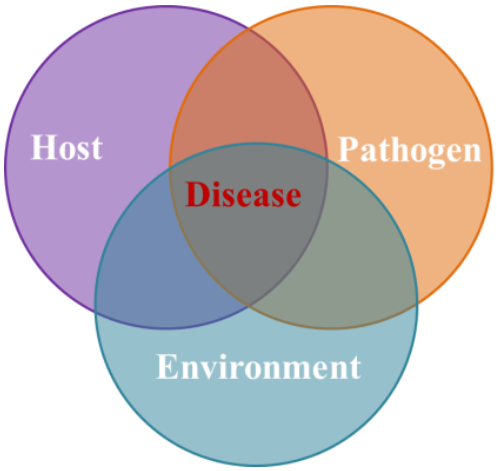

Fig. 1.3: Venn diagram of factors affecting the disease outbreak. Pathogen in presence of susceptible host and suitable environment can cause disease outbreak (adapted from Putignani and Menchella, 2010).

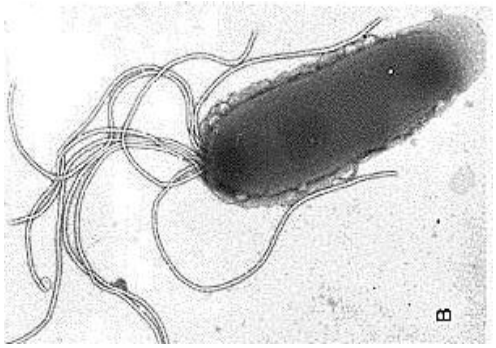

Fig. 1.4: Cellular structure of *Vibrio harveyi.* The bacteria have a rod shape and polar flagella.

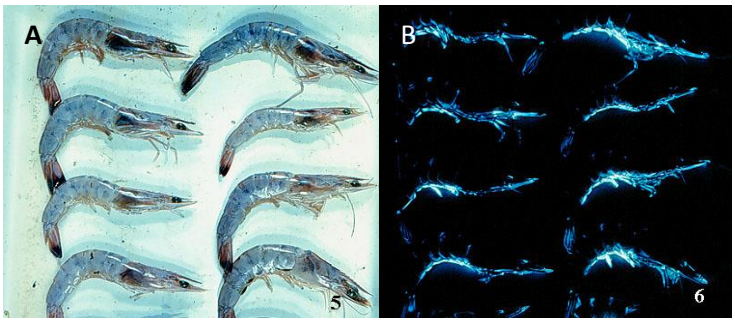

Fig. 1.5: Luminescent vibriosis. The pond-reared (A) healthy *Penaeus vannamei* juvenile and (B) those infected by a luminescent strain of *Vibrio* spp. Vibriosis is a fatal disease in marine organisms that contributes to heavy annual economic losses. While the *Vibrio* infection is systemic, the luminescent bacteria are brightest is the parts where there is more oxygen available (Mohney, 1996). Signs of disease include septicemia, partial tail cramping, lethargy, tissue and appendage necrosis, slow metamorphosis and growth, body malformation, bioluminescence muscle opacity and melanisation (Aguirre-Guzmán et al., 2004).

Although almost all types of cultured animals can be affected by these bacteria, the most serious problems have been reported in penaeid shrimps (Austin and Zhang, 2006; **Fig. 1.5**). Very recently, early mortality syndrome (EMS), a serious emerging disease of cultured shrimp caused by a strain of *V. parahaemolyticus* (Tran et al., 2013), has severely affected the cultured shrimps *P. monodon*, *P. vannamei* and *P. chinensis* (FAO, 2014). Examples of the impact of such diseases include: EMS outbreak in Viet Nam, where about 39 000 ha were affected in 2011. Malaysia estimated production losses of US$ 0.1 billion (2011); while Global Aquaculture Alliance estimates indicated US$ 1 billion. In Thailand, reports from private sector enterprises indicated annual output declines of 30 – 70 %. The disease has been reported in China, Malaysia, Mexico, Thailand and Viet Nam (FAO, 2014). Disease impacts, be it vibriosis or other bacterial diseases, are often adverse both socially and economically because the affected species could be important for human consumption and food security.

Strategies to control diseases

To overcome the negative impacts of the pathogenic biotic and/or abiotic environmental factors on the welfare and economic production of shrimps and other aquaculture species, appropriate management strategies need to be sought. The FAO at its recent Global

Aquaculture Conference concluded that to properly manage the aquaculture diseases and for sustainable aquaculture development, new control strategies are (urgently) needed, with emphasis on prevention than cure (Sorgeloos, 2010). Currently there is an advanced knowledge on biotechnological and genetical tools, and a growing interest in the application of these tools in the development of robust and disease-resistant aquaculture animals.

Selective breeding – a strategy to develop robust animals

Genetically-based host resistance is an attractive characteristic for the farmers and fish/shrimp breeders as apart from investment for the resistant genetic stock, they do not have to make further major outlays for chemical or medical treatment in case of pathogen outbreak. To this end, the breeders select the organisms based on the required phenotypes for several generations. Cross breeding of these selected individuals result in the production of progenies with targeted trait. However, in such breeding programs, there is always a possibility of inbreeding depression especially when the pedigree is not sufficiently monitored or when effective population sizes are too small. Also disease-resistant genes might be linked with other genes on the same chromosome and therefore result in negative regulation of other desirable phenotypes, such as low food conversion ratio, fast growth and etc. (Cock et al., 2009). At present, different breeding programs are being applied for producing healthy and robust fish/shrimp (seeds). Although these approaches are valid and feasible they require large investments as they are very labor intensive. It has therefore, become imperative to find novel ways, which are effective in terms of both cost and time, to produce healthy and disease resistance fish/shrimps for sustainable aquaculture production.

Trans-generational epigenetic inheritance – a new approach to robust animals

Very recently, epigenetic has been considered as a strategy for producing healthy and stress-resistant animals. Epigenetic is the study of heritable changes in gene expression and function that cannot be explained by changes in DNA sequence (Bird, 2007). The

exact molecular bases of these epigenetic changes are slowly being discovered. These types of changes can activate, reduce or completely disable the activity of particular genes. There is mounting evidence that epigenetic changes can be inherited across generations (see chapter 2 for details), however, in aquaculture the concept is rather new and mostly at experimental level.

Model organism for epigenetic studies

Like other invertebrates, shrimps lack adaptive immune system. However, recent evidences suggest that the innate immune system of invertebrates can be primed by various immune stimulants, enhancing its overall response to e.g. a pathogen. Also new findings suggest the presence of an adaptive-like immune system by which priming of the innate immune system of invertebrates establishes elevated or prolonged response to the same pathogens (reviewed in **Chapter 2**). It has been suggested that such sustained capacity of enhanced immune response can be passed on across generations. Epigenetic modifications are believed to play a vital role in these observed phenotypes. However, since the life cycle of e.g. penaeid shrimp, is approximately 1 year, it is very hard to document such kind of phenomenon in a feasible way. Hence, there is an urge to find a new model organism that could represent, at least to some extent, the shrimp immune system and is easy to culture in laboratory condition. Also for experimental purposes, separating genotypic variability due to DNA sequence difference from DNA epigenetic changes is an extremely challenging task in any given population, except if one of the two phenomena could be suppressed or eliminated. In clonal culture of individuals, genetic variability due to DNA sequence variation is eliminated. Transgenerational inheritance of phenotypes and eventually its underlying epigenetic mechanisms can be explored in genetically identical organisms (Petronis, 2006). In this context, the invertebrate aquatic species, the brine shrimp *Artemia*, represent an excellent test model for epigenetic studies as they can reproduce parthenogenetically and a clonal culture can be obtained easily starting from a single animal. As an adaptation to hyper saline and other unfavorable environmental conditions, *Artemia* female produces several successive broods of cysts

which are metabolically inactive. Under favorable conditions the cysts resume metabolic activity, hatch, the animals grow to maturity and start reproducing within a period of about 2 – 3 weeks in laboratory conditions (Van Stappen, 1996). The cysts, produced in these clonal cultures, can be stored in cold conditions for several weeks without losing viability. *Artemia* are found predominantly in extreme habitats where few animals do exist (Van Stappen, 2002) and the ability of this organism to tolerate a wide range of environmental fluctuations makes it highly plastic and hence an unique model for stress studies (Clegg and Trotman, 2002). These *Artemia* strains are put forward in this PhD as a suitable crustacean model system, thanks to their short generation time, high offspring production, storability of cysts, and ease of culture which allows a broad spectrum of environmental factors and phenotypic responses to be studied. The details for the *Artemia* life cycle and its advantages in relation to the envisaged study are reviewed in **Chapter 2.**

Overall aim

Using *Artemia* as model organism, this thesis aimed at investigating the potential role of epigenetic mechanisms in controlling the development and heritability of desirable phenotypes which are of relevance for aquaculture species.

Thesis outline

To accomplish the aims mentioned above, this thesis was divided into 5 major parts each containing several chapters.

Part 1: Introduction and literature review

- **Chapter 1** gives a general introduction of the problem and the thesis outline.
- **Chapter 2** provides an overview of the current knowledge on epigenetics and the mechanisms involved, the role of environmental factors on transgenerational epigenetic effects and heritability mechanisms, including an overview of reported examples. The chapter also discusses the epigenetic control of the immune system in both vertebrates and invertebrates, and finally concludes by discussing the unique

characteristics that make *Artemia* a new suitable laboratory model for epigenetics research.

Part 2: Environmental stress and transgenerational epigenetic inheritance

- **Chapter 3** (Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model) presents experimental data on the effect of environmental heat stress on the modification of epigenetic marks (i.e. DNA methylation and histone acetylation) and their association with the induction and transmission of robust phenotypes (such as increased HSP70 levels, resistance to lethal heat shock and pathogenic *V. campbellii* challenges) across successive generations.
- **Chapter 4** (Non-lethal heat shock induces HSP70 and HMGB1 protein production sequentially to protect *Artemia franciscana* against *V. campbellii*) deals with the interactive study of environmental heat stress and a pathogenic biotic stress on the induction of HSP70 and of a non-histone protein molecule HMGB1, both at the gene and protein levels. This first study, using the gnotobiotic *Artemia* model organism (GART) system, investigated whether these two molecular chaperones are potentially mediating downstream protection to *Artemia* against pathogenic *V. campbellii*.

Part 3: Epigenetic control of innate immune system in *Artemia*

- **Chapter 5** (Trans-generational immune priming in an invertebrate parthenogenetic *Artemia* to a pathogenic *V. campbellii*) verified the phenomenon of immune priming in the invertebrate by carrying out a transgenerational study using a well-developed host-pathogen laboratory model system, clonal population of apomictic parthenogenetic *Artemia* and its pathogenic bacteria *V. campbellii*.
- **Chapter 6** (Probing the immunological and molecular mechanisms of transgenerational immune priming phenomenon by using invertebrate *Artemia franciscana* and *V. campbellii* as host-pathogen model) is an extension of chapter 5.

This chapter provides comprehensive information on the molecular mechanisms associated with the immune priming phenomenon in the invertebrate *Artemia*. This work describes the potential role of epigenetics in the regulation of this phenomenon.

- **Chapter 7** (General discussion and conclusion) recapitulates the overall findings obtained in the entire thesis, conclusions are drawn and possibilities for future research are proposed.

CHAPTER 2

Review of Literature

Genetics and epigenetics – a historical overview

Over the past centuries, various researchers using different experimental models have been looking for the influence of the environment on the phenotypes and fitness of the organisms (Bossdorf et al., 2008) as well as on formation and the evolution of populations (Hedges, 2002). Amongst all, 'epigenesis' is one of the oldest evolutionary theories which was coined by a Greek philosopher 'Aristotle (384-322 BCE)'. The term 'epigenesis' described the transformation and development of an animal's organs over time. The concept was developed based on the observations made on embryonic development of some animals and in particular the different stages involved in the transformation of an egg to a chick. However, the epigenesis theory was not well accepted during that time (Henn and Pausch, 2003) and soon after was replaced with the theory of 'preformism'. According to this theory, all organisms were created at the same time, and succeeding generations developed from miniature versions of the organisms in forms of homunculi, or animalcules, that existed since the beginning of creation. However, during the late 18th century an extended and controversial debate by scientists resulted in epigenesis eclipsing the long-established preformationist view (Baxter and Wilson, 1976). At the beginning of 19th century, the evolutionary biology was mostly associated with the names of Jean-Baptiste Lamarck and Charles Darwin. The evolutionary theory of Lamarck was based on the possible adaptation of organisms to their environment over time by the use or disuse of certain characteristics (Lamarck, 1806). According to Lamarck, the acquired phenotypes can pass to the subsequent generations and will generate a new population in

long time. The Lamarck theory was considered controversial and not accepted due to lack of evidences. Three decades after Lamarck, in 1859, Darwin established a new evolutionary theory; it proposes that all organisms have common ancestors and the diversification results from a process of natural selection, in which the struggle for existence in harsh environments benefits the survival of the fittest. Almost a decade later, in 1868 Darwin proposed another heredity theory named 'pangenesis'. This theory suggested that all cells in an organism can participate in passing the acquired phenotypes from parental generations to the progenies through shedding minute particles called 'gemmules'. It was hypothesized that these particles could circulate throughout the body and finally gathers in the gonads from where they could transmit to the next generation resulting in transmission of characteristics from parent to offspring. Therefore, any environmental modification resulting in changes in parental gemmules would be transmitted to their offspring (Liu, 2008). Two decays later, in 1886 Gregor Johann Mendel established a new heredity theory using pea plants which led to the development of the prominent theory of 'Mendelian inheritance of phenotypes'. The Mendelian theory gave a new insight to the hypothesis about natural selection and heredity. Even though the physical basis of a gene remained obscure until 1940s. It was at the beginning of the 20th century, that a Danish botanist named Wilhelm Johanssen coined the term 'gene' to describe a unit associated with an inherited trait in plants (Johannsen, 1905). This term was adapted from the word 'pangene' from Darwin's theory of 'pangenesis' and later replaced the word unit of inheritance created by Mendel. In 1918, Ronald Fisher brought together the two theories of Darwin and Mendel and this, in 1930, resulted in the modern synthesis of evolutionary biology (Fisher, 1930). Further research was carried out in the following years to determine the characteristics of gene. It was in the year 1953 that Francis Harry Compton Crick and James Dewey Watson, motivated and assisted by the previous findings of Rosalind Elsie Franklin, discovered that genes are the units of double helix structural molecules named 'deoxyribonucleic acid (DNA)' which became the central dogma of molecular biology (Watson and Crick, 1953). Further studies defined a gene as a molecular unit of phenotypic heredity in living organisms. Until 1957 the plastic

responses of a population to environmental variations were typically thought to be associated with their genetic diversity, with higher levels of genetic diversity providing increased adaptive potentials. This idea originated from the general consensus that most developed phenotypes solely have a genetic basis and are subject to Darwinian natural selection and Mendelian inheritance (Crow, 2002; Holderegger et al., 2006). However, this opinion was debated by the epigenetic landscape theory of Conrad Hal Waddington.

Epigenetic landscape theory

According to Waddington's cell fates theory, cells are considered as marbles rolling downhill through a landscape of branching valleys with each new valley determining a possible fate for the cells (see **Fig. 2.1**). The ridges between the valleys maintain the cell fate once it has been chosen (Waddington, 1957). This theory was named as 'epigenetic landscape' (James and Ferrell, 2010) extended from the blend of words 'epigenesis' and 'genetics'. Waddington's epigenetic landscape is a metaphor for how gene regulation modulates phenotypic development as a consequence of environmental variation. Waddington proposed 'genetic assimilation' as a mechanism that allows certain acquired characteristic to become heritable.

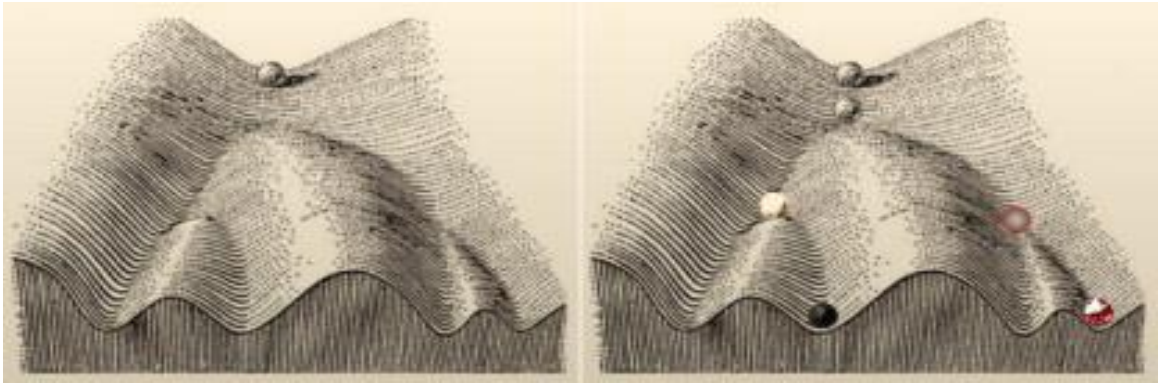

Fig. 2.1: Waddington's classical epigenetic landscape. This landscape represents the process in which the cells make decision during development. Depending on the forces, at different points in this dynamic visual metaphor, the cells that are represented as a balls can choose a specific permitted trajectories, leading to different outcomes or cell fates (adapted from Waddington, 1957).

Genetic assimilation is a process whereby environmentally induced phenotypic variation becomes constitutive, maintained in absence of the initial environmental signal (Pigliucci

et al., 2006). This view was broadened by Nanney (1958) who defined epigenetic as the causes of heritable differences that are not dependent on changes in DNA sequence.

Modern epigenetics - the concept

In the past decade, the genome sequencing projects revealed that complex organisms have lower numbers of protein coding genes than anticipated (Mattick, 2001). For instance, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* appear to have only about twice as many protein coding genes (∼12–14 000) as microorganisms, such as *Saccharomyces cerevisiae* (∼6200) and *Pseudomonas aeruginosa* (∼5500) (Rubin et al., 2000; Stover et al., 2000). Also humans (∼30 000) appear to have only twice as many of the *Drosophila* and *Caenorhabditis* (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001). However, with advances in technology and new annotating techniques, the human genome has shrank to approximately 19000 genes (Juan, 2014). While there is no doubt about the role of alternative splicing in the increased expression of different protein isoforms in higher organism (Graveley, 2001), there is a striking feature of the evolution which was largely overlooked. To date, epigenetic modifications are considered as the alternative source of biodiversity among the organisms. Epigenetic modification has recently been described as an additional source of potential Lamarckian adaptive response (Bossdorf et al., 2008; Johannes et al., 2009) and does not fit in the Darwinian natural selection and Mendelian inheritance theories (Pecinka and Scheid, 2012). By definition, modern epigenetic inheritance refers to mechanisms that permit the development of non-Mendelian, stable transmission of potentially heritable traits to a subsequent generation or generations without any alteration in the DNA sequence (Berger, 2007; Kelly et al., 2010). However, according to Gómez-Díaz et al (2012), epigenetics is defined in two different contexts:

1. Epigenetic plasticity which describes changes in gene expression that occur at the individual cell level, proliferating during mitosis in multicellular organisms and remaining stable during the life span of an individual. Example of such modification is morphologically different castes of bees from genetically identical

individuals through nutritionally triggered DNA methylation (Kucharski et al., 2008).

1. Evolutionary concept which refers exclusively to epigenetic inheritance that is the stimuli-triggered variation in gene expression that is heritable across generations. Example of such epigenetic inheritance is a change in flower symmetry from bilateral to radial in *Linaria vulgaris*, which relates to different levels of methylation of the gene Lcyc (Cubas et al., 1999).

Mechanisms underlying epigenetic modifications

Mechanisms underlying epigenetic modifications involve chromatin remodeling, methylation of cytosine in CpG dinucleotides (often referred to as DNA methylation), histone tail N-terminal modification, and post-translational modification in gene regulation by non-coding/small RNA (ncRNA/sRNA) of certain genes (Lim and Song, 2012; Shankar et al., 2013). In the following section, the role of these mechanisms in regulating gene expression and epigenetic modifications are elaborated.

Non-coding RNA

Previously the only roles considered for RNA was for intermediary carrier of information between DNA and proteins and their involvement in the protein production machinery. However, the more recent findings in field of molecular biology have provided evidence for the role of different RNA species in various cellular functions of an organism. The full review of different RNA species is beyond the scope of this thesis. However, the most relevant examples for epigenetic studies are highlighted.

Small non-coding RNAs are small RNAs that do not directly code for a protein. An accumulating body of evidence suggests that these small RNA molecules of about 20–30 nucleotides with a two-base overhang on the 3' end, have emerged as powerful regulators of gene expression and genome stability (Moazed, 2009). One of the roles considered for these non-coding RNAs is gene silencing by binding to the 3´ UTR of their target mRNA transcripts (Aguilera et al., 2010). The two main classes of these regulatory small RNAs

are microRNAs (miRNAs) and short interfering RNAs (siRNAs). The major difference between siRNAs and miRNAs is that the former can silence their encoding DNA and is considered as defender of genome integrity in response to foreign or invasive nucleic acids such as viruses, transposons, and transgenes, while the vast majority of miRNAs exerts heterotypic silencing and is regulators of endogenous genes (Carthew and Sontheimer, 2009). These small RNAs are processed from larger, double-stranded or hairpin-shaped precursor molecules, by a Dicer enzyme. Dicer enzyme is part of the RNase III family and it cleaves double-stranded RNA (dsRNA) or pre-microRNA (pre-miRNA) into shorter dsRNA fragments of siRNA and miRNA. Dicer enzyme also instigates the RNA-induced silencing complex (RISC), which is a crucial component for RNA interference (Meister, 2013). The catalytic element of RISC is a protein named 'Argonaute', which is an endonuclease capable of degrading messenger RNA (mRNA) by two different pathways (Thomson and Lin, 2009). The eukaryotic Argonaute family is divided into two groups: 1) AGO proteins (also referred to as the AGO clade), and 2) PIWI proteins (also referred to as the PIWI clade). The AGO proteins generally interact with miRNA or siRNAs and are involved in cytoplasmic post-transcriptional gene-silencing procedures (Peters and Meister, 2007; Hutvagner et al., 2008). For silencing a target mRNA, only one strand from double stranded miRNA or siRNA associates with the AGO protein and becomes the guide strand. The strand with the less stably paired 5′ end is preferred for being loaded into AGO proteins (Meister, 2013). Once associated with the effector Argonaute proteins, they form the RISC, which target the complementary nucleic acid sequences (Thomson and Lin, 2009).

On the other hand the main role of PIWI-interacting RNAs (piRNAs) is to protect germline cells from transposons in organisms (Meister, 2013). Depending on the PIWI clade protein that they bind to, the average length of piRNAs can vary between 25 and 33 nucleotides. The biogenesis of piRNAs is independent of Dicer and requires other nucleases (Vagin et al., 2006).

The RNA silencing system is also known to interact with other epigenetic mechanisms. In association with RNA polymerase, RISC can attract or activate DNA

methyltransferases (DNMTs), resulting in DNA methylation (Volpe et al., 2002; Hall et al., 2002; Sigova et al., 2004). This is a known mechanism of siRISC silencing in plants and mammals (Lippman and Martienssen, 2004). These gene expression suppressions occur through target mRNA degradation or translation suppression (Lee et al., 2012). RNAi is also suggested to play a role in the protection of genome against long-term epigenetic defects by re-methylating the specific sequences in order to maintain the wild type methylation pattern (Teixeira et al., 2009). There are accumulating evidences suggesting that RNA mediated epigenetic modification plays a critical role in epigenetic transcriptional gene silencing (Volpe et al., 2002; Hall et al., 2002; Sigova et al., 2004; Wassenegger, 2005; Teixeira et al., 2009).

DNA methylation

DNA methylation refers to the enzymatic addition of a methyl (CH3) group from S-adenosyl-L-methionine (SAM) to the fifth carbon atom of the cytosine (**Fig. 2.2**) or the sixth nitrogen atom of the adenine base (Holliday, 2006)**.** The first identification of DNA methylation (or demethylation) as an important biological modification was described by Griffith and Mahler, who proposed in 1969 that this biochemical addition of a methyl group to DNA provides a basis for long term memory in the brain.

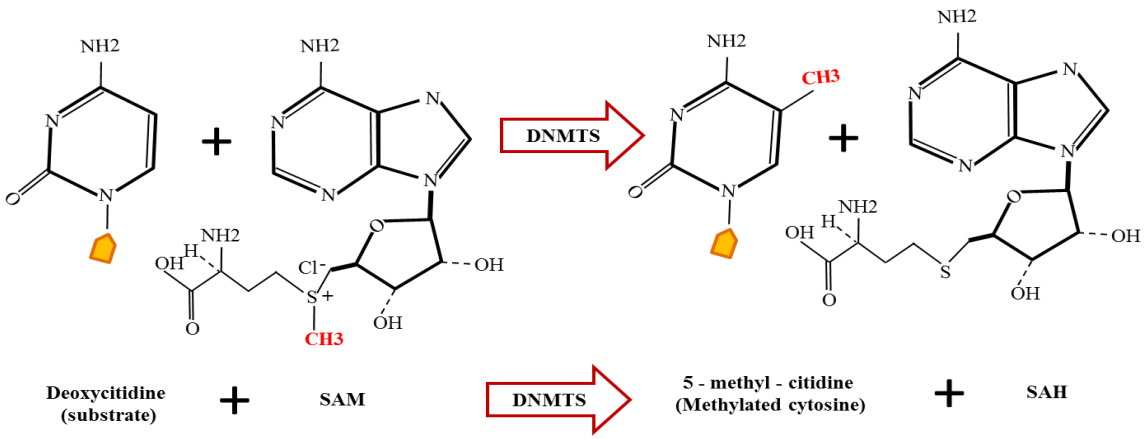

Fig. 2.2: Addition of a methyl group to cytosine. A methyl group is removed from SAH and is added to the 5´ carbon of the cytosine in presence of DNMT enzyme.

The idea was extended in 1975 by independent suggestions from Riggs who outlined a molecular model for the possibility of heritable switching on/off of the genes activity and by Holliday and Pugh who suggested the possible role of DNA methylation in cell proliferation and proliferation maintenance. The conjugation point of the two opinions was the sequence specific and heritable addition of the methyl group to DNA which is catalyzed by group of enzymes known as 'DNA methyl transferases (DNMTs)'.

DNA methylation on the gene promoter can prevent the binding of transcription factors at their specific DNA sequence. Also DNA methylation in repeat regions such as centromeres is crucial for chromosomal stability, for example in case of chromosome segregation at mitosis (Moarefi and Chedin, 2011). It is also likely to suppress the expression of transposable elements and thus to have a role in genome stability (Jones, 2012). Therefore, DNA methylation is generally associated with suppression of gene expression or maintaining at a silenced state.

However, gene body methylation does not only silence but might even stimulate transcription (Jones, 2012). Recent findings suggest that gene body methylation may even play a role in splicing (Laurent et al. 2010; Shukla et al., 2011). Also in a few occasions the methylation of gene body was associated with activation of gene expression (Bongiorni et al., 1999; Field, 2000). For instance, in the genome of the honey bee *Apis mellifera*, CpG methylation is maintained as an epigenetic mechanism to sustain a sufficient transcription of housekeeping genes, necessary for conserved core biological processes in virtually every type of cell (Foret et al., 2009). In organisms, the levels and patterns of DNA methylation can differ substantially between different species. DNA methylation in eukaryotes can be 0 % like in the nematode worm *Caenorhabditis elegans* which lacks DNA methyltransferase (Bird, 2002) or very low like in the fruit fly *Drosophila melanogaster* which can have between 0.1 % and 0.4 % of the cytosines being methylated. Similar value was recorded for the invertebrate *Artemia* (**Chapter 3**). In the genome of the invertebrates honey bee *Apis mellifera* and the wasp *Nasonia vitripennis*, all the three orthologous of DNMTs are found and CpG methylation has also been observed in several genes, but the overall level of DNA methylation has not been

quantified yet (Wang et al., 2006; Schaefer and Lyko, 2007). The vertebrates, in contrast, have high levels of DNA methylation with approximately 5% of the cytosines being methylated in DNA of mammals or birds and approximately 10% in that of fish and amphibians (Field et al., 2004). This methylation occurs evenly throughout the entire genome. These different methylation levels suggest that DNA methylation may have different functions in different organisms.

It should however be noted that in addition to methylation, the cytosine residues are also hydroxymethylated. This process involves enzymatic conversion of 5-methyl cytosine to 5-hydroxymethylcytosine by ten-eleven translocation (TET) families of proteins (Pfeifer et al., 2013). DNA hydroxymethylation is a recent finding and the roles of such modifications are not yet well elucidated. However, some evidences highlight the role of DNA hydroxymethylation as intermediate in the reaction of DNA demethylation or as a signal for chromatin factors. Detailed mechanism is reviewed by Guibert and Weber (2014).

Even though, modification of DNA has been thought to occur at the 5-methylcytosine (m5C), some studies revealed the biological importance of methylation on other nucleotides such as N6-methyladenine (m6A). Earlier, adenine methylation was believed to be essential only for the viability of several bacteria (Stephens et al., 1996; Kahng and Shapiro 2001; Julio et al., 2001; Reisenauer and Shapiro, 2002). However, some evidences suggested that the presence of m6A is not limited to bacterial DNA but also occurs in eukaryotic cells, such as *Paramecium aurelia* (Cummings et al., 1974), *Arabidopsis thaliana* (Ashapkin et al., 2002) and even mammalian cells, such as mouse and rat (Kay et al., 1994; Reyes et al., 1997).

Histone modifications or histone code

Chromatins are the long chains of genomic DNA and associated proteins (**Fig. 2.3**). One of the primary roles of chromatin is to coil the DNA into a smaller volume for fitting in the nucleus of the cell through nucleosomes formation. Nucleosomes are the building blocks of chromatin and contain 147 base pairs, coiled two times around an octamer of

histone (H) proteins (two molecules of H2A, H2B, H3 and H4). The linker histone protein H1 (H5) at the outside of the nucleosome serves to further compact the chromatin (**Fig. 2.4**).

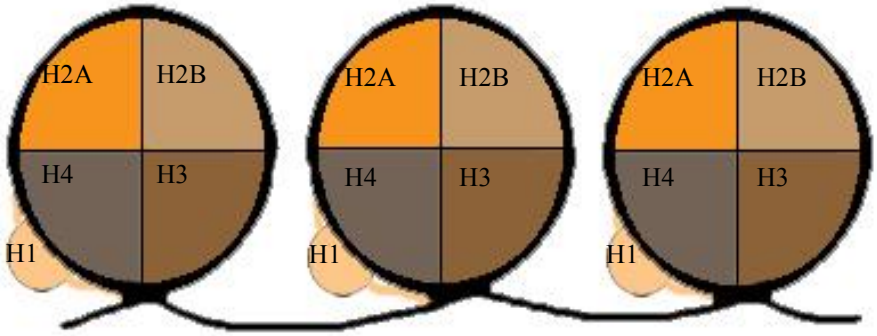

Fig. 2.3: Lateral view of a chromatin structure. Chromatin is a long chain of DNA molecule rapped twice around octamers of histone proteins with the help of linker histone (H1).

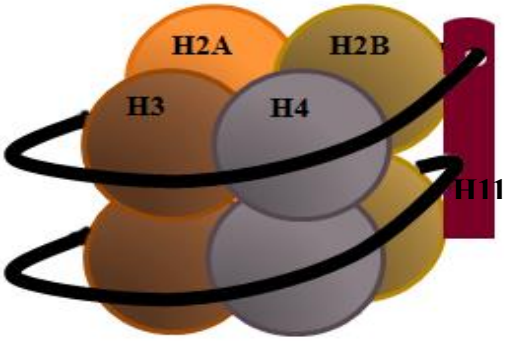

Fig. 2.4: Nucleosome formation. Each DNA-histone unit is called nucleosome.

Till the 1990´s, DNA packing was the only role considered for histones. However, in the past three decades, accumulating evidences suggested that these proteins play significant roles in 1) regulation of gene expression 2) DNA damage repair 3) DNA replication and recombination, and 4) heritable epigenetic regulation (Grunstein et al., 1992; Lennartsson and Ekwall, 2009). Apart from the small globular structure, histones contain a more flexible and charged NH2-terminus also named 'histone tail' that protrudes from the nucleosome and contains 25 – 30 basic amino acids rich residues (Jenuwein and Allis, 2001). The affinity of the histones for each other, for DNA and for

other chromatin associated proteins is determined by post-translational modifications of their protruding amino-terminal tails (Munshi et al., 2009). The covalent modifications (acetylation, phosphorylation, methylation or ubiquitination) on the histone tail can exhibit exquisite variations which in turn regulates the chromatin remodeling and different contacts with the underlying DNA (**Fig. 2.5**; Jenuwein and Allis, 2001).

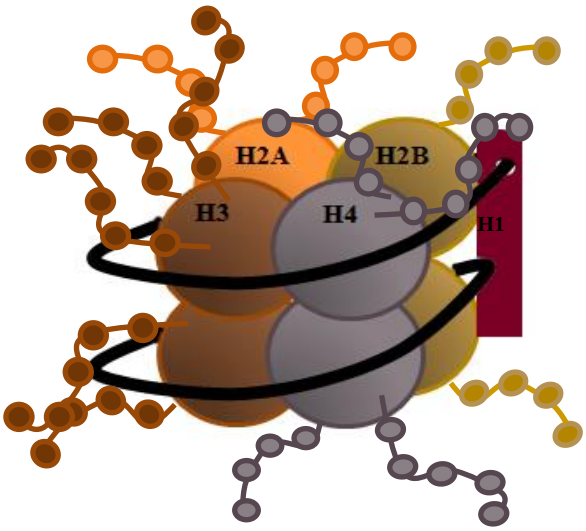

Fig. 2.5: Nucleosome. Each histone has N terminal tails that can be subjected to post-translational biochemical modifications, such as acetylation, methylation, phosphorylation or ubiquitination. Addition or removal of these modifications at specific points on the tails can readily condense or relax the chromatin resulting in reduced or facilitated transcription, respectively.

The distinct histone modifications, on one or more tails, occur sequentially or in combination to form a histone code that is read by other proteins to bring about downstream effects (Strahl and Allis, 2000). Also histone codes can occur differently on different histones and may be transiently altered by the cell environment (**Fig. 2.6**; Bird, 2007). Highly specific enzymes are responsible for the histone tail modifications (Bártová et al., 2008). There are more than 60 detected residuals of the histones where the modifications can take place and yet more are expected to be discovered (Kouzarides, 2007). This infinite array of modifications results in enormous plasticity for functional responses. For example, methylation at lysines or arginines may be in one of three different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines (Kouzarides, 2007). Different histone modifications are associated with different chromatin regulations. For example, acetylation of histones or

di/tri methylation of H3K4 leads to euchromatin (decondensed chromatin) formations, creating conditions for higher gene transcription. Conversely, condensed heterochromatin lacks this histone acetylation and is enriched in methylated H3K9 and H3K27.

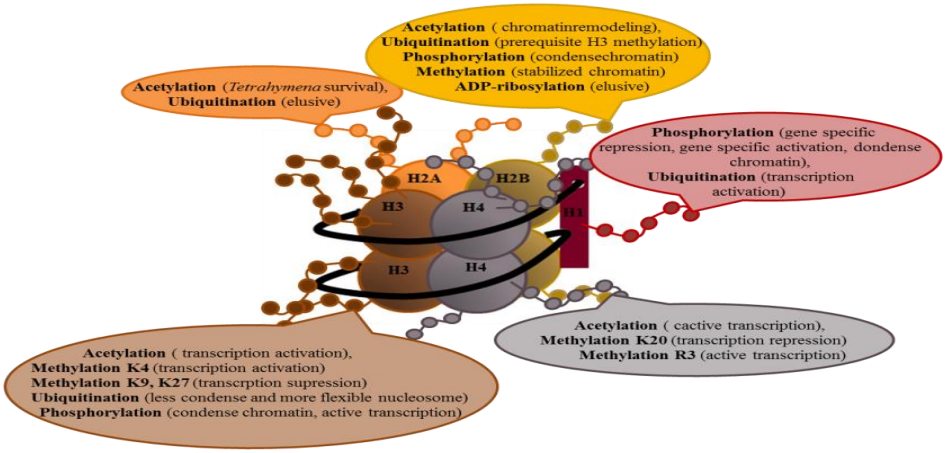

Fig. 2.6: Histone tail modifications. The pictorial representation of a few histone modifications and their biological roles (adapted from Munshi et al., 2009).

Histone methyltransferases (KMTs) and histone lysine demethylases (KDMs) are the enzymes responsible for regulating histone methylations (Nottke et al., 2009). KDMs have a catalytically active site named 'Jumonji' domain (JmjC). The demethylation occurs when JmjC utilizes multiple cofactors to remove the methyl group through hydroxylation. The demethylation by JmjC can be on any of the mono-, di-, and tri-methylated substrates. For other histone modifications two important types of proteins are responsible for specific histone modifications: trithorax group proteins and polycomb (family of proteins that can remodel chromatin) group proteins, which are associated with transcriptionally active euchromatin and transcriptionally silent heterochromatin, respectively (Schuettengruber et al., 2007).

Although histone modification and DNA methylation require different sets of enzymes and are carried out by different chemical and biochemical reactions, there seems to be a biological interaction between the two processes playing a vital role in modulating gene expression programming in the organism (Howard and Yehudit, 2009). Combination of biochemical and genetic approaches demonstrated the possible interaction between the enzymes responsible for generation of histone and DNA modifications (Viré et al., 2006).

In a study where the DNMT was knocked out in mice the histone methylation in certain genomic regions was markedly decreased (Henckel et al., 2009). Interestingly in case of X chromosome inactivation, DNA methylation was considered to play a vital role by initial methylation of the chromosome that was immediately shut off, so that only one chromosome is marked to be silenced (Riggs, 1975). However, recent study on *C. elegans* provided evidences that H3K27 methylation might be responsible for sustaining and stabilizing the formed heterochromatin in the marked X chromosome and has roles in transmitting the memory of chromatin repression transgenerationally and short-term in embryos (Gaydos et al., 2014). Same study provided evidences that polycomb repressive complex 2 (PRC2) promotes long-term memory during development. In another study, DNA methylation was suggested as a secondary epigenetic modification where the DNA methyltransferase enzyme was attracted to the sites where histone H3 is methylated at lysine 9 (Fuks, 2005). Viré et al. (2006) suggested that to methylate the promoter region of certain genes, EZH2 protein which is responsible for H3K27 methylation is required to recruit DNA methyltransferases. In the yeast *Saccharomyces pombe* it was discovered that the RNA induced transcription silencing (siRNA), guides a histone methyltransferase to methylate H3K9, ultimately resulting in heterochromatin formation. This process also takes place in plants and mammals (Wassenegger, 2005). Further research is required to elucidate the interactions and crosstalk between the histone code, the DNA methylation machinery and siRNA.

Epigenetic modifications during cell proliferation and diversification

In multicellular organisms the germline is described as diploid cells that are responsible to pass the genetic information from parents to the progenies. In adults, the germline cells develop into haploid reproductive (gamete) cells by meiotic divisions in the process of gametogenesis. Most complex organisms develop from these very specialized reproductive cells. In bisexual organisms, once the sperm (male gamete) penetrates the egg (female gamete), the single diploid totipotent zygote cell is formed. Totipotent cells are capable of differentiation into all the other possible forms of cells required in an adult

organism. Upon fertilization, a series of epigenetic modifications take place in the parental pronuclei which results in the removal of the gamete-specific modifications to facilitate the ability of embryonic development towards birth (Rivera and Ross, 2013). For this purpose the gamete cells epigenome is erased through a process called 'reprogramming' in order to return the cells to a genetic 'blank state' in which new epigenetic marks determines the faith of the cells (**Fig. 2.7**). For example, in mammals, DNA is progressively demethylated during the pre-implantation states, after which the DNA is re-methylated (Bocock and Aagaard-Tillery, 2009). Still, a very small percentage

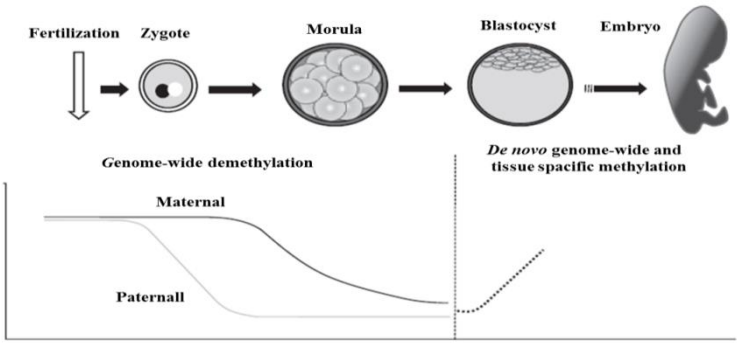

Fig. 2.7: DNA methylation changes during embryonic development. Most of the **e**pigenetic DNA methylation marks are erased during early embryonic development. *De novo*, genome-wide DNA methylation marks are made soon after, during early embryogenesis after which tissue specific epigenetic marks are laid down. The graph demonstrates the methylation process in the maternal and paternal cells after fertilization. Gray line shows the active demethylation of the paternal genome and black line demonstrates the passive demethylation of the maternal genome. Dotted line exhibits the *de novo* methylation evolution at blastocyst stage (adapted from Aguilera et al., 2010).

of genes keep their epigenetic marks through this process and pass unchanged from parents to progenies through the mechanism of self-sustaining feedback loop that was first described in *Escherichia coli* (Novick and Weiner, 1957; Jablonka and Raz, 2009).

Dynamic regulation of oocyte genome is essential for programming the embryo for achieving temporally required developmental landmarks. In the first few days (4 days in human) of embryonic development, more totipotent stem cells are produced by mitotic division of the zygote after which the cells begin to specialize into pluripotent cells. Further the multipotent cells are differentiated from pluripotent cells. The multipotent cells have very limited capability of differentiating compared to the totipotent and

pluripotent cells. Multipotent cells develop into progenitor cells that are programmed to differentiate into multiple, but limited cell types through specific gene activation (Zhou et al., 2011). Therefore, the embryonic stem cells regeneration homeostasis and differentiation require selective activation or suppression of specific transcription programs (Zhou et al., 2011). Thus, synchronized epigenomic modifications are essential for lineage specification and maintenance of cellular identity (Gifford et al., 2013; Zhou et al., 2011; Smith and Meissner, 2013). Interestingly, embryonic stem cells achieve their pluripotent status by locking important regulator genes for future expression, using a polycomb group-mediated repressive histone lock which prevents precocious expression of genes that initiates the differentiation of cells along specific differentiation pathways, but also allows the same genes to be primed for future expression (Spivakov and Fisher, 2007).

Despite of the developmental stability maintained by the stem cells through genetic and epigenetic information, the embryo is highly influenced by the external environment of the parental generation. These transgenerational epigenetic effects can originate either from direct changes within the ancestral germline or by the transfer of information from ancestral somatic cells to the ancestral germline (Devanapally et al., 2015). Environmentally-derived epigenetic changes can be inherited also mitotically through somatic cells which are considered as a potential mechanism by which environmental effects on the epigenome can leave long-term effects on gene expression (Jirtle and Skinner, 2007; Heard and Martienssen, 2014; Devanapally et al., 2015). The ability of the parents for adaptation to the new environmental conditions determines the plasticity of the embryo towards that specific environmental variation. This information is passed on through germline transfer of the information (Devanapally et al., 2015)..

From environment to epigenetic

Apart from epigenetic modifications during the initial development and embryonic stages, environmentally-induced epigenetic modifications can also be an additional source of phenotypic variation. Interestingly, the epigenetically developed new phenotypes in the

parental generation in response to an environmental cue can be inherited to subsequent generations which are not exposed to the environmental factor that caused the change in the parental generation (Aguilera, 2010). Epigenetic inheritance can be multigenerational or trans-generational. Multigenerational epigenetic inheritance is described as modifications that are caused by the direct exposure of the germline to the environmental condition whereas trans-generation effects are valid only if these acquired phenotypes persist in the descendants in absence of the exposed germline (Skinner, 2008). Indeed, the pregnant F0 female carries F1 generation embryos that contain the primordial germ cells creating the F2 generation (**Fig. 2.8**; Youngson and Whitelaw, 2008).

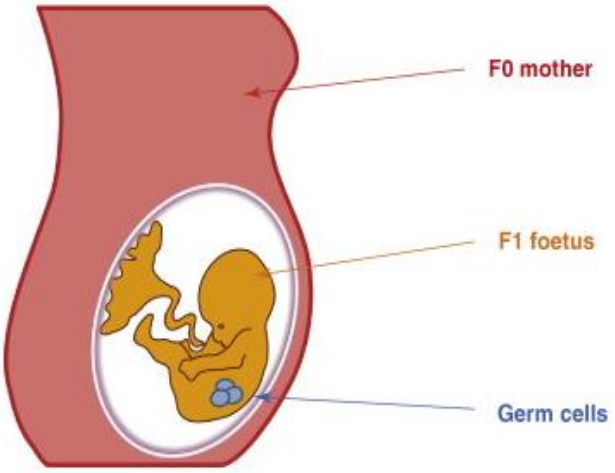

Fig. 2.8: The parental behavior can influence the subsequent generation's phenotypes through germline. An environmental insult during pregnancy to a mother (F0 generation) can manipulate the developing fetus (F1 generation) and the germ cells which will form the F2 generation (Drake and Liu, 2009).

Therefore, at present the trans-generational inheritance of environmentally-induced epigenetic modification is considered authentic only if the acquired phenotypes are still present at F3 generation.

The term epigenetic inheritance is considered to explain both cell–cell and organism–organism transfer of phenotypic information, while trans-generational epigenetics classically refers only to the latter one. Some of the environmental cues that can make epigenetic marks can be nutrition, physiochemical and biochemical environmental variations or even behavior, such as culture, food, smoking, maternal care and depression along with many others. The environmentally-induced epigenetic modifications can

ultimately affect the organism's phenotypes and that of subsequent generations by manipulation of gene expression in order to adapt to new environment. Some examples of environmentally-induced epigenetic modifications and inheritance are described below.

Epigenetic modifications mediated by exposure to toxins

Exposure to chemicals can have epigenetic consequences that may persist for several generations. Toxicants including fungicides, pesticides, or plastic compounds were found responsible for abnormal reproductive or metabolic phenotypes in animal models (Guerrero-Bosagna and Jesen, 2015). These include transgenerational increases in the incidence of obesity, polycystic ovary syndrome (PCOS), pregnancy defects, or germ cell apoptosis that were transgenerationally transmitted in these animals (Anway et al., 2005; Nilsson et al., 2012; Skinner et al., 2013; Ost et al., 2014). In transgenic hamster cell line with inhibited DNMT, nickel (Ni) can condense the chromatin by substituting magnesium (Mg) in the phosphate backbone of DNA (Lee et al., 1995). This phenomenon instigates the condensation of neighboring euchromatins which can be subsequently targeted by *de novo* DNMT enzyme (Lee et al., 1998). The reason that nickel ions target heterochromatin is not yet well understood. However, the reason could be the higher concentration of Mg present in the phosphate backbone of DNA in heterochromatin than euchromatin (Arita and Costa, 2009). Another study conducted by Chen et al. (2006) showed that Ni represses the gene expression by decreasing global histone H4 acetylation and increasing histone H3 lysine 9 dimethylation at the promoter level of several genes in G12 Chinese hamster cell line. The authors suggested that Ni ions interfere directly with Fe (II)-2-oxoglutarate-dependent histone H3K9 demethylase to suppress activation of several genes. Similar changes were reported for other model organisms. For instance, in *Sebastiscus marmoratu,* exposure to waterborne tributyltin or chemical triphenyltin for 48 days decreased the global DNA methylation in dose-dependent manner (Wang et al., 2009). In hens, dietary Cd exposure was shown to hypermethylate DNA by increasing DNMT1 and DNMT3 gene transcription (Zhang et al., 2009). In another study with *Daphnia magna*, Vandegehuchte et al. (2009) demonstrated that Zn exposure of the

parental generation results in stochastic pattern of DNA methylation in 2 subsequent generations.

Behavioral changes and epigenome

Parental mental status or behavior can epigenetically program the offspring phenotypes. It was reported that temporary mental stress caused by maternal separation during the neonatal period (infant) modified the epigenetic status of the glucocorticoid receptor (Gr) promoter in the rat hippocampus. This environmental situation led to changes in gene expression of the pups caused by altered epigenome resulting in persisted abnormal gene expression and behavior throughout life (Weaver et al., 2007). Another example of parental care effects on the offspring phenotype is observed in licking-grooming behavior of maternal rats towards their pups (Weaver et al., 2004). The pups which were licked by their mothers at the beginning of their lives and while growing, exhibited a high expression of glucocorticoid receptors in the hippocampus compared to the pups from low-licking grooming mothers. This modification was reported to be due to different methylation at a single CpG site in the glucocorticoid receptor promoter which resulted in the development of licking grooming behavior of the progenies whose mother licked those. In a similar study, Champagne et al. (2006) reported that the promoter region of the alpha subunit of estrogen receptor was hypermethylated in the hippocampus of low-licking grooming pups, and these changes resulted in suppressed expression of these receptors. Also in the mice, maternal's grooming and licking behaviors towards pups induced epigenetic changes that modifies the animals stress response in the adulthood (Weaver et al., 2004; Meaney and Szyf, 2005; Weaver et al., 2006). This maternal care causes increased expression of hippocampal glucocorticoid receptor (GR) through histone acetylation and DNA demethylation which later alters hypothalamic-pituitary-adrenal axis and the stress response of the pups (Weaver, 2007). In a further study on a non-inbred line mice model, it was shown that chronic and unpredictable stress in early life of mice altered behavioral response not only in the stressed animals when adult but also in their successive, unstressed generations (up to generation F3) (Franklin et al., 2010).

Environmental threats and their influence on epigenome

Environmental threats can result in drastic phenotypic shifts in a population by causing epigenetic modifications. In aphids, also known as plant lice, the presence of predators, crowding or other environmental stresses brought a shift in the population from animals with no wing to winged animals. This adaptive switch took place at early stages of development, through unknown epigenetic mechanisms (Feil and Fraga, 2012). Similarly, in *Daphnia* (water flea) species, external stressors, such as water-borne chemicals or predators instigate dramatic morphological changes. In this case, *Daphnia* arms itself with long pointy helmets, tail spikes and neck teeth, on exposure to the predators during development and these acquired phenotypes persist over several generations in the population (Agrawal et al., 1999). These modifications can change the size of the animals two times bigger than the original one which makes it impossible for certain predators to consume them as a prey (Lloyd et al., 2012). In case of tobacco plants, the insects attack or treatment with jasmonic acid triggers the plants to accumulate toxic nicotine pool in their vacuoles (Baldwin and Schmelz, 1996) and this protects the plants from subsequent insect attacks and, therefore, serves as a stress memory (Kinoshita and Seki, 2014).

Nutrition and epigenome

In mammals, the early developmental stage is a critical period for establishing and maintaining epigenetic marks (Heijmans et al., 2008). In female golden hamsters (*Mesocricetus auratus)*, early food deprivation resulted in sex ratios shifts of their first descendants with lower numbers of males popping up in the population as compared with the normal fed controls. Apart from sex ratio, there were no (typically) greater weight male pups in the food restricted population relative to their females at birth compared to the control group. However, in later developmental stages, the weight of the restricted animals, both male and females was significantly lower than that of the control. These modifications persisted for 2 other generations (Huck et al., 1987). In rats, the early weaning stress led to increased susceptibility of the animals at the later adult stages and their normal weaned offsprings to stress-induced ulcer (Skolnick et al., 1980). Mouse diet

also determines the animals coat color through methylation-sensitive expression of the metastable agouti viable yellow (A^{vy}) allele and shows meiotic epigenetic inheritance (Morgan et al., 1999; Cropley et al., 2006). Hypermethylation of A^{vy} locus resulted in brown or 'pseudoagouti' and hypomethylated A^{vy} locus resulted in a yellow coat color. Nutritional influence on the epigenome and subsequently on the phenotypic changes has been reported for various organisms. For instance, in the honey bees (*Apis mellifora*), female bees can be sterile workers or fertile queens. Both the females are developed from genetically identical larvae but only the ones fed with royal jelly develop into queens. The exact mechanisms behind such transformation are not yet well understood. However, *de novo* methyltransferase DNMT3 is suggested to bring about differential DNA methylation and the differential expression of many genes between queen and worker larvae (Kucharski et al., 2008; Lyko et al., 2010). Another example for the role of nutrition in epigenetic modifications is Dutch hunger winter (1944 – 1645). Ravelli et al. (1976) showed that the male individuals whose mothers were exposed to famine during the last trimester of pregnancy and the first months of life were less obese than controls, whereas exposure in the first half of pregnancy resulted in higher obesity rates than in controls. Later, Heijmans et al. (2008) showed that individuals who experienced famine at the prenatal stage during the Dutch Hunger Winter exhibited decreased DNA methylation on their imprinted (phenomenon in which a gene's epigenetic state is determined by its parental origin) IGF2 gene, 6 decades after the incidence, compared to the non-exposed ones from the same sex siblings. Interestingly, examination of the F2 generation by Veenendaal et al. (2013) demonstrated higher weights and body mass index (BMI) in adult offspring of prenatally exposed F1 fathers compared to the offspring of unexposed ones. However, this effect was sex-specific and the offspring of prenatally exposed mothers did not exhibit these phenotypes. Another relevant example of nutritional effects on multigenerational epigenetic inheritance of acquired traits was reported by Benyshek et al. (2006) in the rat model (non-inbred lines). These authors demonstrated that impaired glucose metabolism in F1 rats exposed to a nutritional stress during gestation persisted through maternal transmission to the F3 generation.

Other physiochemical environmental variations

Besides the environmental factors described above, epigenetic modifications also happen to occur by means of various other physiochemical environmental variations. For instance, spaceflight environment has been shown to induce heritable epigenetic changes by alteration in cytosine methylation patterns and activation of transposable elements in the plant rice *Oryza sativa* (Ou et al., 2009). Spaceflight is a complex environment with cosmic radiation, microgravity and space magnetic fields with their interactions. A study with apomictic dandelions demonstrated that the environmental stressors such as salinity, pH or nutrients can significantly modify the pattern of DNA modification on specific genes. Interestingly the authors found that methylation pattern was stressor specific and was inherited for two successive generations (Verhoeven et al., 2010). Similar results were obtained from other plants in response to nutrient limitation, salt stress and the application of plant hormones (Feil and Fraga, 2012). Also in human, adverse environmental conditions in early developmental stages is considered to result in increased risks for disease in the adulthood (Waterland and Michels, 2007). Using a genetically identical *Arabidopsis thaliana* line plant model, Whittle et al. (2009) demonstrated that plants exposed to mild heat (30°C) treatment in the parental and F1 generations exhibited markedly improved fitness (5-fold increase in seed production per individual) in a later generation (F3). In this experiment the increased heat-specific fitness phenotype was preserved for 2 generations (F2 and F3) none of which were treated with mild heat. This result led to the conclusion of an environmentally-induced epigenetic and heritable adaptive phenomenon. These examples illustrate that altered phenotypes induced by environmental stress may be transmitted across multiple generations.

Interestingly, heat stress is also associated with epigenetic regulation of sex determination in various fish species. For example, Navarro-Martín et al. (2011) demonstrated that increased temperature (from 15°C to 21°C) in the environment of European sea bass juveniles, mediates the hypermethylation of gonadal aromatase (cyp19a) enzyme that is responsible for irreversible conversion of androgens into estrogens. In normal conditions the male juveniles of European sea bass have double the

DNA methylation levels in promoter region of cyp19a compared to the females. Therefore, the elevated temperature increased the chance of induced-masculinization of females through DNA methylation-mediated control of aromatase gene expression.

Environmental biotic challenges

In an environment, living organisms are surrounded by different species of micro-organisms including pathogenic bacteria and viruses, and they have a significant evolutionary impact on the host fitness and life history (Anderson et al., 1980; Boots and Begon, 1993). Host-pathogen interactions are amongst the highly plastic, dynamic and competitive system interactions (Gomez-Diaz et al., 2012). Upon a pathogen attack, healthy cells of the host impose selective constrains to restrict or eliminate the threats. Therefore, many pathogens evolved developing an extreme level of phenotypic plasticity in order to cope with the pressures imposed by the host (Moore, 2002; Fernandez-Morera et al., 2010). In the same way, the host phenotype is drastically modified by the presence of a pathogen which in some cases can be even inherited by subsequent generations (Poulin and Thomas, 2008). A good example of such adaptation is reported by Adamo (1999) in crickets (*Acheta domesticus*) exposed to pathogenic bacteria (*Serratia marcescens*) or in parasitic larvae of the parasitoid fly (*Ormia ochracea*). The author found that the females which were injected with either the pathogenic bacteria or the cell wall from the same bacteria exhibited high productivity in terms of numbers of laid eggs. This phenotype was not observed in the animals challenged with the parasite. In another study, with citrus trees, Martini et al. (2014) demonstrated that bacterial pathogen, *Candidatus Liberibacter asiaticus* (Las), modifies the odors released by the plants. This phenotypic modification attracts more *Tamarixia radiate* which is the citrus tree parasite and the pathogenic bacteria vector (Martini et al., 2014). In case of houseflies (*Musca domestica*), the infectious fungus *Entomophthora muscae* can be eliminated by the animals immune system only if the flies can find warm enough temperatures during the first 3 days post infections. Therefore, the infected animals seek warmth during this phase. If they can't find such habitat, the fungus takes the lead and changes the fly's

phenotypic behavior to instigate them for moving to cold areas where fungal spore formation is favored (Watson et al., 1993). The swift modifications accruing during the host-pathogen interactions and co-evolution leave no doubt about interference of epigenetic modifications in this entire process (Gómez-Díaz et al., 2012).

Epigenetic control of immune system

The immune system of both vertebrates and invertebrates has nearly unlimited capacity to respond to environmental triggers. Two types of the immune response, innate and adaptive, provide a comprehensive defense against environmental hazards and also eliminate nonfunctional or malignantly transformed cells.

The adaptive immunity is characterized by immunological memory and specificity and it provides the immune system with the information required for responding to each new interaction with the microbial world on the basis of past experiences with the same (Shelby et al., 2003; McFall-Ngai, 2007). To initiate and maintain the required adaptive immune response, rapid activation of lymphocytes is necessary (Sainte-Marie, 2010). Lymphocytes are responsible for mediating both innate (natural killer cells) and adaptive (T and B cells) responses in vertebrates (Van Furth, 1969). In order to maintain control over adaptive immunity, many immune genes are highly and strictly transcribed (Sospedra and Martin, 2005). Therefore, epigenetic modifications are believed to play an essential role in the orchestration of gene regulation during adaptive immune responses. Cellular and humoral immunity are the two arms of adaptive immune system. Cellular immunity involves activation of T cells, which in turn can stimulate B cell mediated production of antigen specific antibodies. T or B cells can also eliminate the antigens independently. As an example, in vertebrates, antigen encounters results in activation of the innate immunity which later instigates multipotent CD4+ T cells differentiation into distinct T cells populations, including T helper (Th) 1 which promotes cell mediated immunity and clears intracellular pathogens, Th2 that is parasite fighters, and Th17 which fights against fungi and bacteria (Placek et al., 2009). Epigenetic modifications are involved in the differentiation of CD4+ T cells to inducible regulatory T cells (iTreg)

after the immune system is challenged with other stimulus (Sakaguchi et al., 2008). Histone modifications and nucleosome positioning are considered to have key roles in specific gene regulation in T lymphocytes (Roh et al., 2006). Also DNMT3a mediates the regulation of the Th1 and Th2 development (Gamper et al., 2009) which further instigates specific immune responses through specific production of distinct sets of cytokines which epigenetically regulate, sustain and control the immune responses (Fernández-Morera et al., 2010).

B cells are another important component of the adaptive immune system and important effectors of humoral responses in vertebrates. In antigen encounters, the B cells, upon induction by the T helper cells, differentiate into antibody-secreting plasma cells. The B cells produced antibody destroys bacteria, viruses, and even tumor cells by activation of complement and/or interaction with lytic cells resulting in antibody dependent cell-mediated cytotoxicity. The entire process of the B cells production, induction and activation is epigenetically regulated through activation of certain genes (Fernández-Morera et al., 2010). In both cases of immunity with B or T cells, the dead cells are engulfed by innate immune cells like macrophages.

Unlike adaptive immunity, the innate immune system includes components that are constitutively present and are instigated immediately upon exposure to triggering factors mainly the conserved pathogen-associated molecular patterns (PAMP's), as for example lipopolysaccharides (LPS) from bacteria and β-1,3-glucans from fungi, and/or damage associated molecular patterns (DAMPs, such as heat shock proteins, high mobility group box 1 proteins), through a series of pattern recognition receptors (PRRs) (Jiravanichpaisal *et al.*, 2006). Amongst the PRRs, the Toll-like receptors (TLRs) are the best characterized ones (Akira et al., 2006; Hajishengallis and Lambris, 2011). In vertebrates, a foreign molecule encounter triggers the TLRs innate immune responses by promoting signaling pathways through activation of adaptor TLRs mainly myeloid differentiation 88 (MyD88) which sequentially activates the release of proinflammatory cytokines, such as interleukins, type I interferon, chemokines and antimicrobial peptides (Qian and Cao, 2012). During this process, nuclear factor kappa-light-chain-enhancer of activated B (NF-

κB) signaling or mitogen-activated protein kinases (MAPK) are also activated which in turn up regulates the transcription of innate immune-related genes. NF-κB is responsible for mounting the innate or adaptive inflammatory responses whereas MAPK instigates only the innate immunity. In invertebrates, Toll pathway is activated by Gram-positive bacterial or fungal pathogens while the Gram-negative bacteria activate immune deficiency (IMD) pathway (Lemaitre et al., 1996). Unlike vertebrates, in invertebrates the TLRs does not detect the pathogens directly and they are instigated by upstream receptors (Michel et al., 2001; Gobert et al., 2003; Pili-Floury et al., 2004), that are required for cleavage and activation of ligand for Toll which is the C-terminal 106 amino acids of the protein spätzle (SPZ) (Weber et al., 2003; Hu et al., 2004). The SPZ modification further activates the NF-κB pathway. The details of these pathways are reviewed elsewhere (see review, Kaneko and Silverman, 2005). In a Gram-negative bacteria encounter, peptidoglycan (and exceptionally Gram positive *Bacillus* spp. which have amidated *meso* – dia minopimelic acid, DAP) can potentially activate the IMD pathway (Lemaitre et al., 1997; Leulier et al., 2003; Kaneko et al., 2004). IMD is considered as a Death Domain protein which is very similar to the mammalian receptor interacting protein 1 (RIP1) (Georgel et al., 2001) that is responsible for activation of NF-κB pathway. The activation of the IMD pathways and the factors responsible are previously reviewed (see Kaneko and Silverman, 2005; Waterhouse et al., 2007) and are beyond the scope of this review.

Unlike vertebrates which possess both arms (innate and adaptive) of the defense system, the host defense in invertebrates is believed to rest entirely on the innate immune mechanisms, (theoretically) lacks specific memory to remember the previous encounters with the pathogens (Shelby et al., 2003). The invertebrate innate immune system is divided into humoral and cellular defense responses. Humoral response is characterized by the production of antimicrobial peptides (AMPs), reactive intermediates of oxygen or nitrogen and complex enzymatic (e.g. phenoloxidase, peroxinectin) cascades that regulate clotting or melanization of the hemolymph (Beutler, 2004). Cellular response, in contrast, include several steps: first there is chemotaxis, or active migration of hemocytes; followed by opsonization, or secretion of soluble factors (AMPs) which act directly on

the microbial membrane against a broad range of microorganisms (Bachère 2003; Beutler 2004; Donaghy et al., 2009), followed by phagocytosis (the internalization and degradation of foreign material) and 'oxidative or respiratory burst'; during respiratory burst a rapid release of reactive oxygen occurs (superoxide radical and hydrogen peroxide) and immune cells use NADPH oxidase to reduce O_2 to oxygen free radicals and then H_2O_2. The immune effector molecules (proteins and peptides) are found in the hemolymph, and immune cells (hemocytes) are found both in the hemolymph, and in certain tissues, for example the gills (Gross et al., 2001).

The conviction that invertebrates lack specificity and memory was strongly supported by the fact that they do not possess antibodies, or T or B cells. However, this dogma that adaptive (anticipatory) immunity is absent from invertebrates is now cracking, because at least some invertebrates were reported to have immunological specificity and memory of a kind that is a functional equivalent of adaptive immunity in vertebrates (see Kvell et al., 2007 for a review). However, to set it apart from the 'memory' known in vertebrates, the 'memory'-like phenomenon in invertebrates are termed 'immune priming' (Schmid-Hempel, 2005).

Epigenetics and immune priming phenomenon in invertebrates

The immune priming phenomenon has been broadly defined as induction of increased resistance in an organism following previous exposure to a pathogen or an immune elicitor, such as pathogens, pathogen associated molecules or even stressors that can activate the immune system (Schmid-Hempel, 2005). This mechanism has already been demonstrated in plants (Paulert et al., 2010) and a high degree of specificity to microbial compounds has slowly been discovered in the invertebrates (Schulenburg et al., 2007). There is a report that has attempted to identify the underlying mechanisms involved in priming in invertebrates using the *Drosophila-Streptococcus pneumonia* host-pathogen model (Hauton and Smith, 2007). Results indicate that the phagocytes are responsible for immunity and that their activity is primed by the exposure to an initial sub lethal dose. However, it is not clear from these data whether this is due to increased functioning of

existing phagocytes, or to some other aspect of the immune system that have not been characterized yet. In the invertebrate woodlouse *Porcellio scaber*, hemocytes showed increased phagocytosis of a previously encountered bacterial strain compared to other bacteria after priming with heat-killed bacteria (Roth and Kurtz, 2009). Their data suggest that specific immunological protection can be induced by a single exposure to a low dose of heat-killed bacteria, thus resembling the phenomenon of a vaccination. A few studies have indicated that the immune priming effect in invertebrates can extend for days and weeks, sometimes for almost the lifetime of the adult (Moret and Siva-Jothy, 2003; Korner and Schmid-Hempel, 2004). Other studies have indicated that similar to vertebrate adaptive immunity, the immune priming phenomenon in invertebrates are transgenerational i.e., the protective/immune responses are transmitted from parents to offspring (Sadd et al., 2005; Tidbury et al., 2011; Freitak et al., 2009). For example, in *Daphnia magna*, offspring from mothers primed with the bacteria *Pasteuria ramosa* suffered less of a reduction in fitness, in terms of reproductive output, when subsequently infected with this bacterium (Little et al., 2003). This acquired protective effect was also found to be specific, such that offspring exposed to the same parasite strain as their mother had a greater fitness advantage than offspring exposed to a different parasite to their mother. Despite a few phenomenological observations indicating that invertebrates possess a certain degree of specificity and memory similar to a kind of vertebrate adaptive immunity, there is no comprehensive and clear report on the underlying mechanisms behind immune priming phenomenon in invertebrates. However, there is evidence indicating that epigenetic regulatory mechanisms are a central element in immune system function, allowing an appropriate gene expression pattern in immune cells in response to internal or external environmental cues, including pathogenic factors (see review Fernández-Morera et al., 2010). For instance, epigenetic modifications have been shown to underlie information storage during innate immune memory in both plants (Jaskiewicz et al., 2011) and mammals (Chen et al., 2014; Saeed et al., 2014). It is possible that epigenetic regulation/modification of the immunological pathways are the mechanistic

basis for immune priming phenomenon in invertebrates. Further studies are warranted to verify this hypothesis.

Model organisms for epigenetic studies

Several animal species have been developed as models to investigate various aspects of mammalian/non-mammalian biology, for instance developmental and cell biology, evolutionary biology, immunology, and genetics. The most popular animal models are the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the zebra fish *Danio rerio*, the African claw frog *Xenopus laevis*, the chicken *Gallus gallus domesticus*, the mouse and the rat *Rattus norvegicus* (Hau and van Hoosier, 2005; Cogburn et al., 2007; Jenner and Wills, 2007; Kennedy, 2008; **Fig. 2.9**).

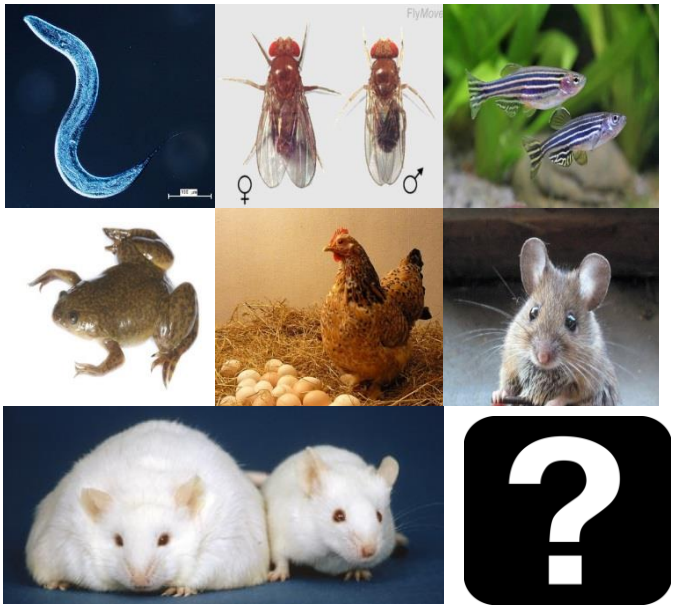

Fig. 2.9: Some examples of animal models that are used for epigenetic studies. The question mark points at the possibility to discover and describe organisms that can serve as excellent models for epigenetic studies.

These model organisms have some characteristics in common, such as easy culture, non-specialist living requirements and flexibility for experimental manipulation, but also have one or more special features that qualify them to be an outstanding subject of

research. For instance, in *C. elegans* the fate of each of the constituent cells can exactly be traced, and in *D. melanogaster* mutations can easily be induced and the resulting phenotypes are easily observed. These model organisms have provided critical breakthroughs in understanding various fundamental questions in biology. Benefiting from technological advancements, the genome sequences of various model organisms have been obtained. However, our knowledge about how the information encoded in their genomes is regulated or interpreted is still limited. Thus, understanding epigenetic phenomena has become a major focus of research activity in the current post-genomics era.

Epigenetic studies also necessarily require specific experimental models. In fact, various model organisms have been used to date, through which a wealth of knowledge has been acquired from several landmark epigenetic discoveries (Allis and Reinberg, 2007). Different model organisms offer different advantages, and all are vital for learning about the processes and mechanisms involved in epigenetic modifications regulation. For instance, multiple species of yeast have been used as model systems to study chromatin structure (Allis and Reinberg, 2007). Because DNA methylation is not detected in yeast, the presence of DNA methylation in the fungus *Neurospora crassa* (**Fig. 2.10**) made it a model organism for DNA methylation studies and contributed to the discovery of repeat-induced point mutation (RIP), which is considered as genome defense mechanism (Allis and Reinberg, 2007).

Fig. 2.10: ***Neurospora crassa*** **fungus.**

Histone variants, the first histone acetyltransferase, histone lysine methylation, histone phosphorylation and one RNAi pathway were discovered in the protozoan *Tetrahymena thermophila* (Allis and Reinberg, 2007; **Fig. 2.11**).

Fig. 2.11: The protozoan *Tetrahymena thermophile.*

The studies on the fruit fly *D. melanogasterhave* led to the discovery of chromatin remodeling and histone modifying proteins (Lecuyer et al., 2007). Plants, such as *Arabidopsis thaliana* (**Fig. 2.12**), have epigenetic mechanisms as sophisticated as those of mammals, including RNAi pathways, DNA methylation, histone modification, and chromosome remodeling complexes (Henderson and Jacobsen, 2007).

Fig. 2.12: *Arabidopsis thaliana.*

Finally, mice, as mammals, are genetically more similar to humans than any of these model systems and are used as models for epigenetic research, particularly in embryology, stem cell research and environmental studies, including the effects of behavior and nutrition on epigenetic states.

Despite the fact that these animal models provided researchers with a great variety of information on epigenetics, they have limitations at least in one way or another. For example, rodents often show strong behavioral variation even in standardized environments; and zebra fish, chicken and frog can be difficult to maintain in a laboratory because of their long generation time (see review Yan et al., 2014). In addition, the mode of reproduction in eukaryotic organisms selected for the epigenetic studies has always been a topic of debate. The issue gets more attention when the trans-generational epigenetic inheritance is concerned (Kelly et al., 2010). Many of the model organisms are bisexuals where the male and female contribute to the genetic and epigenetic characteristics of their offspring. In heterogeneous wild populations of bisexual animals the observed phenotypes could be due to genotype selection rather than epigenetic modification. Therefore, the selected genotypes can contribute to the observed phenotypes in the progenies rather than epigenome interference. To address this issue, the inbred lines are produced to unify the genotype of the population and stabilize the presence of the epialleles in all the individuals (Johannes et al. 2009; Reinders et al., 2009; Richards, 2009). However, in this context inbreeding depression can be a barrier for continuing with trans-generational studies. Also a study conducted by Vergeer et al. (2012), using perennial plant *Scabiosa columbaria* (**Fig. 2.13**) as model organism, demonstrated that inbreeding can lead to increased evidence of hyper methylation in the inbred population and this new phenotype significantly contributes to the inbreeding depression.

Fig. 2.13: ***Scabiosa columbaria.***

Interestingly, artificial demethylation of the plants by germinating seeds on filter paper saturated with a daily-refreshed 50 mM 5-azacytidine nullified the negative effects of inbreeding. However, this treatment cannot be applied if the aim of the experiment is to determine different patterns of DNA methylation in response to particular treatments. Also, as mentioned earlier, in bisexual organisms, many of the epigenetic modification are reset during meiosis and only selected epigenetic modifications can find their way to the next generation by self-sustaining feedback loops which are metabolic pathways through which different pattern of activity can be maintained (Bond and Finnegan, 2007; Jablonka and Raz, 2009). Removal or retention of these modifications is individual dependent which can dilute the acquired epigenetic marks in the population. However, it is also true that the bisexual animals offer the possibility to investigate in particular the maternal or paternal inheritance of the phenotypes, selection studies or X chromosome silencing patterns (**Fig 2.14**).

Fig. 2.14: Calico cat. The patchy distribution of color on the body of calico cats results from the random inactivation of one X chromosome in females (Ahn and Lee, 2008).

From the point of transgenerational epigenetic inheritance, the asexual mode of reproduction becomes an interesting criterion for the model organisms to be used in epigenetic studies. In asexual reproduction the offspring are the progenies of a single organism from which they inherit their entire genotype. Asexual mode of reproduction does not include meiosis therefore, except for the case of automixis where the cells go through meiosis and there is chance for recombination, the progenies genotype is the exact copy of the parent. Thus, most studies have modeled asexual reproduction as clonal

(Balloux et al. 2003; Bengtsson 2003; Halkett et al. 2005). This characteristic allows for the production of a clonal population from a single parent, and this provides the opportunity to study epigenetic modifications and their pattern in response to certain treatments in absence of genotypic diversity.

In higher organisms excluding plants, the asexual mode of reproduction is described by the term 'parthenogenesis'. The common forms of parthenogenesis include arrhenotoky which produces haplodiploid progenies where haploid males develop from unfertilized eggs and diploid females develop from dipoloid fertilized eggs; deuterotoky which includes the development of either males or females from unfertilized eggs; and thelytoky which is the complete form of parthenogenesis where the unfertilized eggs develop into diploid females which creates a population of females progenies (Gavrilov and Kuznetsova, 2007). Based on this new classification, the previously used terms, such as 'obligatory' and 'facultative' parthenogenesis are not considered since in some of the parthenogenetic populations the males can be produced but do not contribute into progenies.

Apomixis is the mode of thelytoky parthenogenesis where the progenies are all female and clone of the mother. For epigenetic studies, apomixes seems to be a promising mode of reproduction where meiosis and recombination do not interfere and the entire populations of progenies are clonal females. Such reproductive behavior minimizes genetic variation in the experiments on transgenerational epigenetic inheritance. Considering all these features suitable animal models need to be selected to study the phenomena of epigenetic and in particular trans-generational epigenetics.

Artemia as an emerging model organism for epigenetic studies

The brine shrimp (*Artemia*) belonging to the phylum Arthropoda and class Crustacea is found worldwide in hypersaline environments, such as inland salt lakes (some of which at > 4500 m altitude) and coastal salt pans. *Artemia* are well-established live feed for the marine fish and shrimp larvae in the aquaculture industry (**Fig. 2.15**) but at the same time

offers a variety of benefits that makes it an excellent model organism for biological researches.

Fig. 2.15: *Artemia* naupli (larvae) used as feed for feeding various species of fish/shellfish larvae. At this stage the motility of *Artemia* nauplii attracts the larvae to predate and get fed on this nutritious aqua-feed. Also the small size of the nauplii matches the small size of animal's mouth.

Being an invertebrate excludes *Artemia* from the long list of organisms with ethical issues. It has several other advantages that makes it an excellent model for epigenetic studies. *Artemia* is one of the most ancient species that first appeared ~400 million years ago (Dattilo et al., 2005) which is considered as an evolutionary advantage for this organism. *Artemia* is considered as an extremophile with a wide range of salinity (from 3 to 300 ppt) and temperature (from 15 to 55 °C) tolerance. They can even survive in fresh water for a maximum period of about 5 h (Vos et al., 1980), but cannot reproduce in it (Treece, 2000). In natural environment, *Artemia* can even tolerate high oxygen fluctuation (Clegg, 2007). These characteristics make this crustacean an exceptional model organism for plasticity and evolutionary studies since different adaptations, distinctive genetic features and marked biogeographic patterns are all found in the organism (Athanasios et al., 2006).

In nature, there exist several hundreds of *Artemia* strains with a diversity of phenotypic characteristics. These strains can be classified into seven sexual and one asexual species based on their mode of reproduction (Triantaphyllidis et al., 1998; Van Stappen, 2002) with bisexuals including *A. franciscana, A. persimilis, A. salina, A. urmiana, A. sinica, and A. tibetiana* and parthenogenetic *Artemia* species which

reproduce through apomixes. In *Artemia* parthenogenesis is considered to be evolved independently from the sexually reproducing species at least 3 million years ago (Baxevanis et al., 2006) and unlike the closest species to *Artemia* (i.e. *Daphnia*) the mode of reproduction is not environmentally regulated. Like in most of the Arthropods, sex determination system in *Artemia* is WZ/ZZ with females being heterozygote. Bisexuals are diploid with 2n = 42 (except *A. persimilis* where 2n = 44), with polyploid parthenogens ranging from 2n to 5n (Abatzopoulos et al., 1986). The two mode of reproduction provides the *Artemia* several added advantages. It is because in the context of (transgenerational) epigenetic research, parthenogenetic *Artemia* can generate clonal offspring, which offers a unique opportunity to separate genetic and epigenetic influences on the phenotype, an invaluable asset when studying epigenetics. Additionally, from an epigenetic inheritance viewpoint, *Artemia* also offers a number of other major experimental advantages, such as it has a very simple and short generation time of about 14 – 28 days, females ovulate every 140 h with high fecundity. It possesses two independent reproductive pathways that allow it to produce either dormant cysts by oviparous reproduction under unfavorable environmental conditions or swimming larvae ovoviviparously under favorable conditions (Sorgeloos, 1980; Van Stappen, 1996). To cope with harsh and complex habitats such as salt lakes, *Artemia* are able to release their offspring into a dormant, encysted state, when the environmental conditions become adverse, in place of releasing swimming nauplii. This strategy ensures *Artemia* offspring survival. Under unfavorable environmental conditions, the embryological development is interrupted at the gastrula stage; the embryo is surrounded by a chitinous shell and released in the environment as diapausing cysts through oviparity (Van Stappen, 1996; **Fig. 2.16**). The unfavorable environmental conditions include high salinity, low oxygen levels, short days, or extreme temperature variation (Clegg and Trotman, 2002; Nambu et al., 2004). These dormant cysts will remain at diapause until they are exposed to diapause breaking triggers, such as desiccation, dehydration, cold or chemical treatment. After the diapause break, once the environmental condition is optimum and stable, the cysts resume

their development (Lavens and Sorgeloos, 1980; Drinkwater and Clegg, 1991; Nambu et al., 2008).

Fig. 2.16: *Artemia* cyst. Cysts size of *Artemia* can vary between 200 - 250 µm. After collection, the cysts are dehydrated and can be preserved for several weeks, months and even more than 1 year.

The produced cysts can be stored for several weeks without losing viability and can be hatched on demand within 24 h. The cysts are also a powerful gene bank that allows preserving the genetic memory of the parental population. The attractiveness of *Artemia* as a potential (transgenerational) epigenetic model organism is further enhanced by the fact that the storability of cysts produced in each successive generation allows for 'common garden' experiments, i.e. the simultaneous testing of animals from subsequent generations, a convenient property if one seeks to minimize environmental interference in experiments (Vanschoenwinkel et al., 2010). *Artemia* nauplii become adult (**Fig 2.17**) and starts reproducing in about 22 -28 days (**Fig. 2.18**). The unique characteristics of *Artemia* life cycle may cause additional differences in terms of germline exposure to the environmental treatments since *Artemia* nauplii do not carry the uterus. The animals start their sexual maturity once they are at the juvenile stage. At present it unknown if the exposure of germline in parental generation nauplii (to certain environmental stressors) would contribute into the F2 generation animal's germline exposure. As this information for *Artemia* is unknown, in the current thesis the information available for mammals (as demonstrated in **Fig. 2.8**) were considered for epigenetic inheritance of phenotypes.

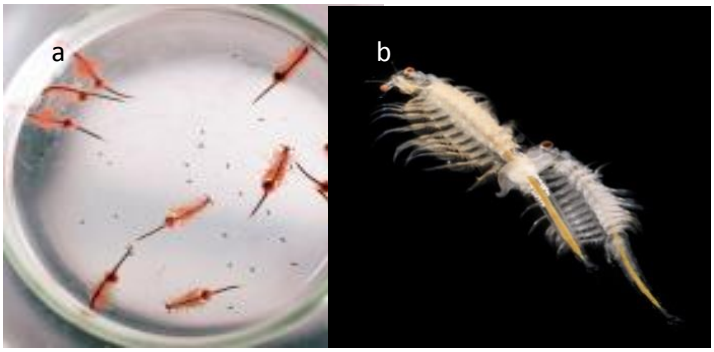

Fig. 2.17: (a) Adult parthenogenetic *Artemia* (a) and bisexual *Artemia* (b).

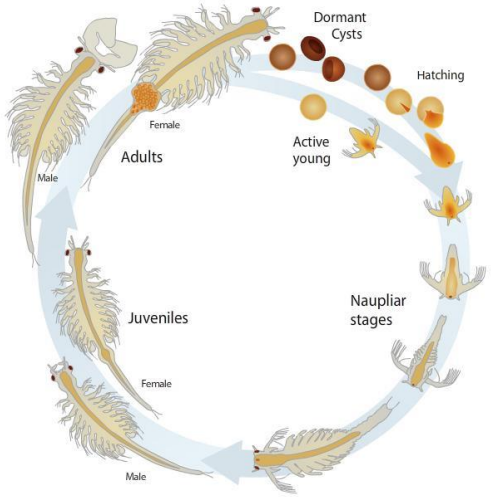

Fig. 2.18: *Artemia* life cycle from cyst to adult. *Artemia* possesses two independent reproductive pathways that allow it to produce either dormant cysts by oviparous reproduction under unfavorable environmental conditions or swimming larvae ovoviviparously under favorable conditions. The nauplii produced either oviparously or ovoviviparously molts several time and becomes adult over a period of 14 - 28 days (for explanation see the text; Source: University of Utah, Genetic Science Learning Center).

In addition, the *Artemia* genome has been sequenced and annotated by an UGhent effort (De Vos, 2014), giving easy access to genes of interest. Interestingly, this sequencing effort has produced close to one million SNPs. As the *Artemia* genome sequence is available now, it allows to investigate genes involved in (epi)genetic traits. Also the sexual mode of reproduction in *Artemia* provides the opportunity to study the paternal or maternal inheritance of phenotypes. A landmark experiment using wild population of bisexual *A. franciscana* has demonstrated that it is possible to produce 12 generations of *Artemia* under mass selection, imposing selective environmental stress, in

combination of whole genome re-sequencing. This allows for identifying of genes that are displaying allele shifts, related to the imposed selective conditions. In this respect, *Artemia* has a significant upside potential as a gene discovery platform for crustaceans, which might help to speed up breeding efforts and phenotype development.

PART 2

Environmental Stress and Transgenerational Epigenetic Inheritance

Swimming against the flow of a river needs strength and courage. Otherwise even a dead fish can move with the flow of the water.

- Mahmoud Hessabi (1903 - 1992)

CHAPTER 3

Environmental Heat Stress Induces Epigenetic Transgenerational Inheritance of Robustness in Parthenogenetic *Artemia* [1]

Abstract

The notion that phenotypic traits emerging from environmental experiences are heritable has long been debated. However, the recent report of non-Mendelian transgenerational epigenetic inheritance i.e. the inheritance of traits that are not determined by the DNA sequence might make such a phenomenon plausible. In our study, by carrying out common garden experiments, we could provide clear evidences that, upon exposure to non-lethal heat shocks, a parental population of parthenogenetic (all female) *Artemia* (originating from one single female) experiences an increase in the levels of HSP70 production. Interestingly, this acquired phenotypic trait was transmitted to three successive generations, none of which was exposed to the parental stressor, resulting in their increased 1) tolerance towards lethal heat stress, and 2) resistance against pathogenic *Vibrio campbellii*. This transgenerational inheritance of the acquired traits was associated with altered levels of global DNA methylation and acetylated histones H3 and H4 in the heat-shocked group compared to the control group, where both the parental and its successive generations were reared at standard temperature. These results indicated that epigenetic mechanisms, such as global DNA methylation and histones H3 and H4 acetylation have particular dynamics that are crucial in the heritability of the acquired adaptive phenotypic traits across generations.

[1] Norouzitallab P, Baruah K, Vandegehuchte M, Van Stappen G, Catania F.J, Vanden Bussche J, Vanhaecke L, Sorgeloos P, Bossier P (2014). FASEB Journal. 28(8):3552-3563.

Introduction

Living organisms, both terrestrial and aquatic, are constantly influenced by a multitude of environmental (abiotic and biotic) stressors to which they react with a battery of responses (Kültz, 2003). The plastic responses of a population to environmental insults are typically thought to be associated with its genetic diversity, with higher levels of genetic diversity providing increased adaptive potentials (Crow, 2002; Holderegger, et al., 2006). This idea originated from the general consensus that most phenotypes determining stress tolerance have a genetic basis and are subject to Darwinian natural selection and Mendelian inheritance (Pecinka and Scheid, 2012). While there is no doubt about the validity of these principles, epigenetic modification has recently been suggested as an additional source of potentially adaptive response (Bossdorf, 2008; Johannes et al., 2009). Epigenetic inheritance refers to mechanisms that permit the development and stable transmission of potentially heritable traits to a subsequent generation or generations without any alteration in the DNA sequence (Kelly et al., 2010). This non-genetic transgenerational transfer of phenotypes is regulated by numerous mechanisms, such as DNA methylation, histone modifications and post-transcriptional gene regulation by non-coding RNA (Lim and Song, 2012; Shankar, et al., 2013). Epigenetic modifications can directly be influenced by environmental cues, potentially allowing an organism to respond and adapt to environmental fluctuation over a short timescale (Feil and Fraga, 2011; Pecinka and Scheid, 2012; Suter and Widmer, 2013; Vandegehuchte and Janssen, 2013). The notion that acquired traits induced by environmental cues could become heritable dates back to Lamarck and has been controversial ever since (Jablonka and Lamb, 2002; Holliday, 2006). Over the past century, various researchers using different experimental models have attempted to demonstrate the direct influence of the parental environment on the phenotypes and fitness of the offspring (Ng et al., 2010; Verhoeven, et al., 2010; Ismaeil et al., 2013). However, other authors demonstrated that stress-induced epigenetic modifications are not always transmitted to subsequent non-exposed generations (Boyko et al., 2010; Verhoeven and van Gurp, 2012). At present, there is little evidence in the literature which unequivocally demonstrates transgenerational epigenetic

inheritance of environmentally-induced phenotypes (Paszkowski and Grossniklaus, 2011; Pecinka and Scheid, 2012). Indeed, many studies failed to exclude parental effects, could not or did not demonstrate the persistence of traits across generations, were not sufficiently replicated to distinguish stochastic effects from treatment or did not integrate phenotypic characterization with more detailed molecular and genetic analysis (Pecinka and Scheid, 2012; Grossniklaus, et al., 2013). Studies systematically examining such a large range of generations under stress and control conditions are still scarce, but crucial to understand if and how environmental conditions can induce heritable phenotypic changes, potentially through epigenetic inheritance.

Selection of an appropriate animal model that allows to better delineate the contribution of epigenetics to the inheritance of acquired traits is of high importance. The apomictic parthenogenetic *Artemia*, an aquatic invertebrate, represents an exceptional model for studying transgenerational epigenetic inheritance. It has a short generation time and is able to parthenogenetically generate clonal offspring, a feature that minimizes genetic variability in the experiments. Moreover, this invertebrate possesses two independent reproductive pathways that allow it to produce either dormant eggs (cysts) by oviparous reproduction under unfavorable environmental conditions or swimming larvae ovoviviparously under favorable conditions (Sorgeloos, 1980; Van Stappen, 1996). The produced cysts can be stored for several weeks without losing viability and can be hatched on demand. The cysts are also a powerful gene bank that allows preserving the genetic memory of the parental population. Moreover, the storability of cysts allows for 'common garden experiments', i.e. the simultaneous testing of animals from subsequent generations (Vanschoenwinkel et al., 2010), a convenient property if one seeks to minimize environmental interference in experiments.

Among the environmental stressors animals can encounter, pathogen attack and heat stress are quite common. In addition, heat stress is expected to increase in frequency in the coming decades as a consequence of climate change (Ahuja et al., 2010). Upon exposure to heat stress, animal's cells express a family of proteins termed heat shock proteins (HSPs), whose function is to protect the cells from stress-induced damages by

maintaining protein biogenesis and homeostasis (Muchowski and Wacker, 2005; Baruah et al., 2012). In our previous study, we have demonstrated that exposure of *Artemia* to a non-lethal heat stress at 37 °C for 30 min followed by 6 h recovery induced HSP70 production within the animals and conferred protection against subsequent lethal stress (41 °C for 20 min) or pathogenic vibrios (*Vibrio campbellii*, *V. proteolyticus*) (Sung et al., 2007; Baruah et al., 2012). Additionally, in another study, it was shown that heat shock induces a series of epigenetic modifications, providing evidence for the existence of a heat stress-related histone code (Fritah et al., 2009).

Here, using a population of parthenogenetic *Artemia* obtained from a single female, we investigated the effects that environmental stressors can have on the emergence and transgenerational inheritance of phenotypic traits in *Artemia*. After daily exposure of a parental population of parthenogenetic *Artemia* to non-lethal heat shocks at early life stages, we assessed the phenotypic and epigenetic changes in the treated population and in three subsequent (untreated) generations. We found that, upon exposure to non-lethal heat shocks, parental *Artemia* experience an increase in the levels of HSP70 production. This acquired traits was transmitted to three successive generations, none of which was exposed to the parental stressor, resulting in their increased 1) tolerance towards lethal heat stress, and 3) resistance against pathogenic *V. campbellii*. The transgenerational inheritance of the acquired phenotypes was associated with significantly altered levels of global DNA methylation and acetylated histones H3 and H4 in the treatment group compared to a control group, where both the parental and its successive generations were reared at standard temperature.

Materials and methods

Culture of single female offspring

A population of apomictic parthenogenetic *Artemia* was obtained ovoviviparously from a single tetraploid female of Megalon Embolon saltworks in Greece (Baxevanis and Abatzopoulos, 2004). The animals were grown till adult in 35 g/L artificial seawater under controlled laboratory conditions (constant temperature 28 °C, light intensity 27

μmol/m^2s for 24 h per day, adequate aeration). Throughout the culture period, the animals were fed ad libitum everyday with live green micro algae *Tetraselmis suecica* obtained from the Culture Collection of Algae and Protozoa Department (CCAP) (Dunstaffhage Marine Laboratory, Scotland, UK). Dead animals were removed daily, and complete water exchange was carried out twice a week.

Artemia cysts production and hatching

Under non-optimal environmental conditions, such as high salinity or low oxygen, *Artemia* switches from an ovoviviparous to an oviparous mode of reproduction. To induce the *Artemia* to produce cysts, the salinity of the culture water was gradually increased from 35 to 80 g/L over a period of 10 days. Deposited cysts enter in a state of diapause and are not ready for hatching (Drinkwater and Clegg, 1991; Nambu et al., 2008). To terminate cyst diapause, the collected cysts were dehydrated in a saturated NaCl brine solution, and exposed to a hibernation of three months at -20 ºC, and then stored as activated cysts at 4 °C until use. For hatching, the activated cysts were washed with sterile distilled water to remove the brine, rehydrated in sterile distilled water for 1 h, and then transferred to a 1 L glass bottle containing 35 g/L sterile artificial seawater. Following incubation at 28 °C under constant illumination for 48 h, the emerged instar II larvae (stage at which mouth is open for ingestion of food) were used for experimental treatments.

Daily non-lethal heat shock treatment of the parental (F0) generation

A group of 1800 instar II larvae was distributed in two groups (treatment and control), each with 3 replicates. Each group was maintained in a 2 L glass bottle containing sterile artificial seawater (35 g/L), maintained at 28 °C under constant illumination (approximately 27 μmol/m^2/s) and aeration. For daily hyperthermic treatments, *Artemia* larvae were exposed to two non-lethal heat shocks starting from day 2 post hatching and continued for 14 days ahead of the reproductive period (under standard laboratory conditions, *Artemia*'s uterus develop on day 16 post hatching). Stress exposure at early

life stages was applied to ensure that the uterus carrying the cysts/embryos was not directly exposed to the experimental stress conditions. The first heat shock was given at 35 °C for 30 min followed by a recovery period of 75 min at 28 °C. The second heat shock was applied at 38 °C for another 30 min. For this purpose, the nauplii from the 2 L glass bottles were collected over a sieve (250 μm), rinsed several times with sterile seawater to reduce bacterial load and then re-suspended in 500 mL glass bottles that contained sterile artificial sea water (stocking density, approximately 4 animals/mL) maintained at 28 °C. The animals were exposed to a non-lethal heat shock at a Δt rate of 7 °C/min in a preheated and controlled water bath system with thermostat heaters accurate at ± 0.01 °C. Heat-shocked *Artemia* were slowly acclimated back to 28 °C (Δt = 3 °C/min). After the second heat shock, animals were collected again over sieves, rinsed with sterile seawater, and then resuspended in the 2 L bottles containing 1.8 L of sterile seawater (28 °C). The control group, cultured isothermally at 28 °C, went through the same handling process. This regime of non-lethal heat shocks was established based on preliminary studies (results not shown).

Production and collection of the progenies as nauplii and cysts

Approximately 23 days post hatching, adult females started producing larvae (F1 generation). The F1 larvae from 3 replicates were isolated, pooled and further cultured isothermally at 28 °C to maturity, after which the F2 larvae were collected. The experiment was continued until, and including, the F3 generation. The adult parental (F0), F1 and F2 animals (from day 28 post hatching) in both control and treatment were induced to produce F1, F2 and F3 cysts, respectively by gradually increasing the salinity from 35 to 80 g/L over a 10 day period. An aliquot of the larvae collected during each generation was subjected to thermal and *V. campbellii* challenge tests as described below (see section, thermotolerance and *V. campbellii* challenge assays). This entire procedure of non-lethal heat shock treatment of the F0 generation followed by offspring production and challenge assays was repeated once to confirm the reproducibility of the results (see appendix C). The detailed experimental design is shown schematically in **Fig. 3.1**.

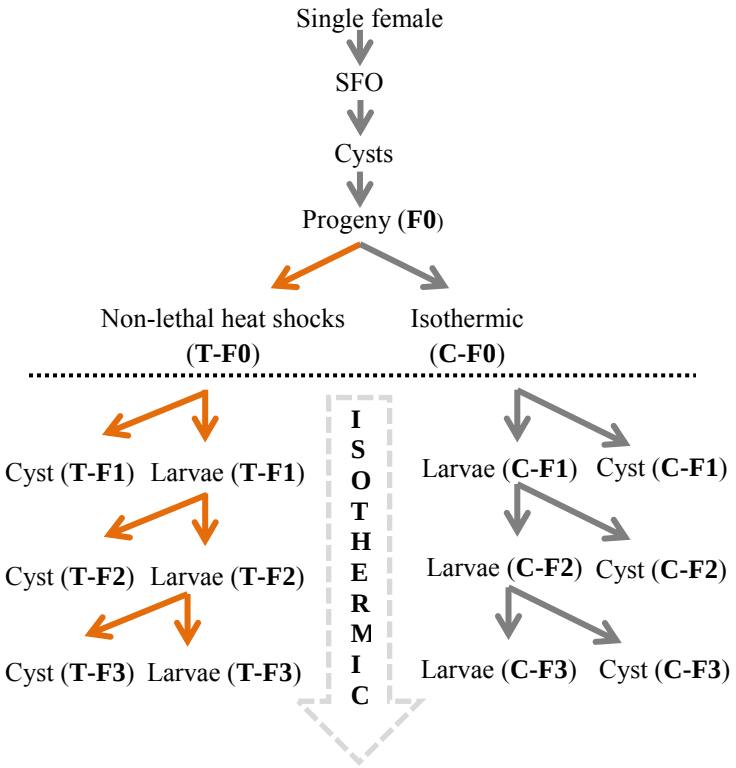

Fig. 3.1: Scheme of the experiment. A single female parthenogenetic *Artemia* was propagated to the next generation (i.e., single female offspring, SFO), under normal growth conditions. The SFO (all females) was reared normally until adult, which were then induced to produce cysts (F0). Upon hatching, the F0 progeny were divided into two groups. One group was exposed daily to two non-lethal heat shocks (the first at 35 °C for 30 min followed by 75 min recovery period at 28 °C and the second at 38 °C for another 30 min) starting from day 2 post hatching until day 15 post hatching i.e., ahead of the reproductive period (T-F0). The other group was grown isothermally at 28 °C (C-F0). Approximately, 23 days post hatching, the parental (F0) females from the treatment (T-F0) and control (C-F0) groups produced their next generation larvae i.e., T-F1 and C-F1, respectively. The F1 larvae from both groups were further cultured isothermally at 28 °C to maturity, after which the F2 larvae were collected. The experiment was continued until, and including, the F3 generation. The adult parental (F0), F1 and F2 *Artemia* in both the control and treatment were induced to produce F1, F2 and F3 cysts, respectively by gradually increasing the salinity from 35 to 80 g/L.

Thermotolerance assay

Artemia larvae (instar II stage) collected from each generation (F1 to F3) were rinsed several times with sterile (artificial) seawater and then resuspended in 35 g/L sterile seawater (28 °C). Groups of 20 larvae were transferred in 7 replicates into separate sterile 40 mL glass tubes that contained 30 mL of 35 g/L sterile seawater (28 °C). The larvae were challenged with a lethal heat shock at 42 °C for 15 min (Δt = 7 °C/min) and then

transferred to 28 °C. Resistance to this thermal shock was determined by counting the live nauplii at every indicated time interval (Clegg et al., 2000).

Vibrio campbellii challenge assay

Vibrio campbellii strain LMG21363, stored in 40 % glycerol at -80 °C, were incubated at 28 °C for 24 h on Marine Agar 2216 (Difco Laboratories, Detroit, MI, USA) and then grown to log phase in Marine Broth 2216 (Difco Laboratories, Detroit, MI, USA) by incubation at 28 °C with shaking. The bacteria were transferred to a sterile tube, centrifuged at 2200 × *g* for 15 min at 28 °C, suspended in filtered (0.2 μm) sterile seawater, and immediately used in challenge experiments. Bacteria cell numbers were determined spectrophotometrically at 550 nm according to McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/mL. Groups of 20 *Artemia* nauplii (from each generation) were transferred in 7 replicates into separate 40 mL glass tubes containing 30 mL of 3.5 g/L sterile seawater. They were then challenged with *V. campbellii* at 10^7 cells/mL (Baruah et al., 2013). The survival of *Artemia* was scored at every indicated time intervals.

Common garden test

For the experiment, age and size-synchronized larvae were used, which were obtained by hatching the oviparously produced cysts as described previously (Briski et al., 2008). Briefly, *Artemia* cysts from the control and treatment groups collected from all the generations during the experiment were hatched simultaneously by incubating in 35 g/L sterile (artificial) seawater at 28 °C for 48 h. The required number of emerged larvae was used for thermal and *Vibrio* challenge assays as described above. The remaining number were further reared isothermally (in triplicates) till juvenile stage. Once at juvenile stage, animals were sampled, pooled, rinsed in sterile distilled water, immediately frozen in liquid nitrogen and stored at -80 °C for analysis of epigenetic marks.

Protein extraction

Artemia juveniles (16 days old, 18 individual) were homogenized by rapid agitation with a required amount of 0.5 mm diameter glass beads in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) (Baruah et al., 2013) containing 10% protease inhibitor cocktail (Sigma Aldrich, Belgium). Subsequent to centrifugation at 4000 x *g* for 5 min at 4 °C, supernatant was collected and protein concentration was determined following the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Total histone extraction

Histone extraction of *Artemia* samples (16 days old juveniles, 18 individuals) was carried out using the EpiSeeker histone extraction kit (ab113476, Abcam, Cambridge, UK) according to the manufacture's instruction. Histone concentration was determined following the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Western blot analysis of HSP70 and histone H4 total acetylation

Histone or protein samples were combined with loading buffer, vortexed, heated at 95 °C for 5 min and then electrophoresed in 10% SDS-PAGE gel, with each lane receiving equivalent amounts of protein (10 µg). HeLa (heat-shocked) cells (Enzo Life Sciences, Farmingdale, NY, USA) (6 µg) were loaded in one well to serve as a positive control and for further calculation of the amount of HSP70 and histone H4 acetylation in the samples. Gels were then transferred to polyvinylidene fluoride membranes (Immun-Blot™ PVDF) (BioRad, Nazareth Eke, Belgium) for antibody probing. Membranes were incubated with blocking buffer [50 mL of 1x phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60 min at room temperature. For HSP70 analysis, the membrane was incubated with mouse monoclonal HSP70 antibody, clone 3A3 (Affinity BioReagents Inc., Golden, CO., USA), which recognizes both constitutive and inducible HSP70 (Baruah et al., 2012), at the recommended dilution of 1:5000. Horseradish peroxidase conjugated donkey anti-mouse IgG was used as secondary

antibody at the recommended dilution of 1:2500 (Affinity BioReagents Inc., Golden, CO., USA). For histone H4 acetylation analysis, the membrane was incubated with rabbit polyclonal anti-acetylated protein primary antibody (ab 193, Abcam, United Kingdom), which is sensitive to histone H4 acetylation at the recommended dilution of 1:1000. Horseradish peroxidase conjugated goat anti-rabbit IgG (Gentaur, Kampenhout, Belgium) was used as secondary antibody at the recommended dilution of 1:2500. The membranes were then treated with enhanced chemiluminescence (ECL) reagent (GE Healthcare, UK), with subsequent signal detection by means of a ChemiDoc MP Imaging System (BioRad, Nazareth Eke, Belgium). The relative signal intensity was quantified by densitometry with Biorad Image Lab™ Software version 4.1.

Analysis of histone H3 total acetylation

Histone (5 µg) samples from all the generations (F0 to F3) of the control and treatment groups were analyzed for histone H3 total acetylation using the EpiSeeker histone H3 total acetylation detection fast kit, fluorometric (ab131561, Abcam, Cambridge, UK) according to the manufacture's instruction.

Global DNA methylation

DNA was extracted from 16 days old *Artemia* juveniles (10 individuals) with a ReliaPrep™ kit (Promega, Leiden, The Netherlands) following the manufacturer's protocol. One µg of DNA was enzymatically digested to nucleosides (including 5-methyl-2'-deoxycytidine or mdC) with benzonase (Merck, Darmstadt, Germany), phosphodiesterase I (Sigma, Belgium) and alkaline phosphatase (Sigma, Belgium) according to Quinlivan and Gregory (Quinlivan and Gregory, 2008). Finally nucleosides were separated, detected and quantified on an Accela UHPLC (Ultra-High Performance Liquid Chromatography) system (Thermo Fisher Scientific, San José, CA, USA) with a Waters Acquity HSS (High Strength Silica) T3 column (2.1 x 100 mm, 1.8 µm) coupled to a TSQ Vantage Triple Stage Quadrupole mass spectrometer (Thermo Fisher Scientific, San José, CA, USA) by means of an Heated ElectroSpray Ionisation interface (HESI-II).

The mobile phases consisted of 0.08% aqueous formic acid (A) and 0.08% formic acid in acetonitrile (B). A step-wise linear gradient was used starting from 99% A going up to 90% B. Mass over charge ratios of detected ions (m/z) for mdC were 242.076 (precursor ion) > 126.032 (product ion), for deoxyguanosine (dG) these were 268.058 (precursor ion) > 134.983, 152.021 (product ions). These product ions were used for quantifying the levels of mdC and dG in the digests, based on the analysis of external standard series of (1) mdC in a nucleoside mixture containing dG, dA and T and (2) mixtures of dG, dA, dC and dT. The concentration ratio [mdC]/[dG] was used to calculate the fraction of methylated cytosines in *Artemia* DNA, as described by Vandegehuchte et al. (2009).

Statistical analysis

At each generation, significant differences in the epigenetic marks (DNA methylation and histone H3 acetylation) between the control and the treatment were determined by Student's *t*-test analysis using the Statistical Package for the Social Sciences (SPSS) version 19.0. Survival data were analyzed for statistical differences by subjecting the data to logistic regression analysis using GenStat (VSN international) version 16. Significance level was set at $P < 0.05$.

Results

Progenies of daily non-lethal heat-shocked ancestors have increased level of HSP70 in the three subsequent unstressed generations

We have shown previously that exposing *Artemia* to a non-lethal heat shock induces HSP70 production within the animal (Baruah et al., 2012). Here, we tested whether the phenomenon of non-lethal heat shock-mediated HSP70 induction in parental generation of *Artemia* could be transmitted to its progenies across three subsequent generations. To this end, we carried out Western blot analysis on protein extracts from the (heat-shocked) parental *Artemia* and their isothermally-grown F1, F2 and F3 progenies. We found that exposure of the parental *Artemia* to daily non-lethal heat shocks (T-F0) markedly

increased HSP70 production relative to the non-heat shocked control groups (C-F0, **Fig. 3.2)**.

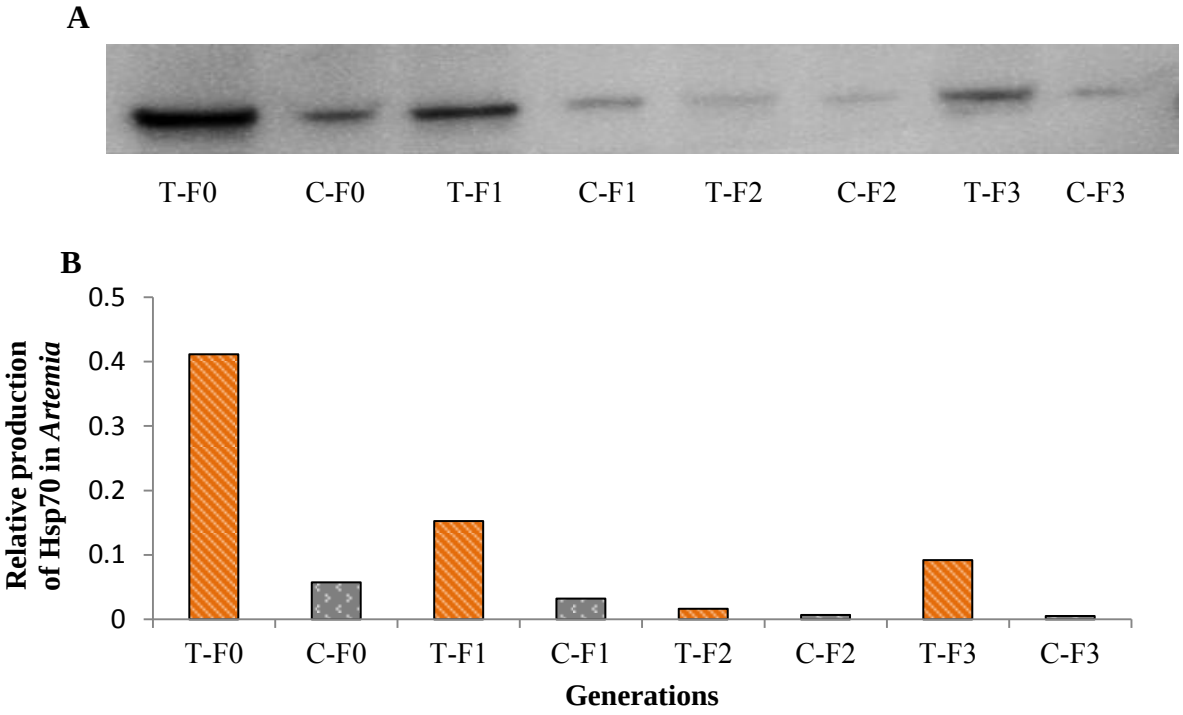

Fig. 3.2: Immunoblot analysis showing HSP70 production level in *Artemia* from the parental generation (F0) and its three successive generation progenies (common garden test). *Artemia* F0 larvae were exposed to either daily non-lethal heat shocks prior to uterus development (treatment, T) or were not treated (control, C). Thereafter, *Artemia* from each group were grown isothermally to produce F1 cysts and larvae. The F1 larvae were further grown isothermally to produce F2 cysts and nauplii, from which F3 cysts were produced. Cysts from F1 to F3 were hatched simultaneously, the larvae produced were grown isothermally till juveniles and sampled. (A) Protein extracted from F0 to F3 juveniles was resolved in SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane and probed with an antibody specific to *Artemia* HSP70. *Artemia* protein (10 µg) was loaded in each lane. HeLa (heat shocked) cells (6 µg) were loaded onto one well to serve as a positive control and for calculating the amount of HSP70 in the sample. (B) Quantitative analysis of HSP70 in *Artemia* is expressed as ng/µg of protein. The results are the values obtained from one biological replicate containing 18 juveniles.

Remarkably, this increased HSP70 level was also observed in the isothermically-reared progenies for three subsequent generations. In these individuals, the level of HSP70 remains consistently higher across generations and appears to fluctuate, initially decreasing (generation F1 and F2) and then spiking (generation F3). More specifically, T-F0 animals that were daily exposed to non-lethal heat shocks exhibited 7.3-fold increase

in HSP70 production level than the respective control (C-F0). This relative increase in the HSP70 level reached 4.6-fold at T-F1 generation and 2.4-fold at T-F2 generation, whereas at T-F3 generation, the level of HSP70 increased once more compared to the control C-F3 progeny by a factor of 17 folds.

Progenies of daily non-lethal heat-shocked ancestors exhibit increased resistance against V. campbellii in the three subsequent unstressed generations

Since elevated HSP70 (or non-lethal heat shock) has been reported to induce resistance against biotic and abiotic stressors in *Artemia* (Sung et al., 2007; Baruah et al., 2010; Baruah et al., 2011), we next investigated whether the non-lethal heat-shocked parental population (T-F0) also could transmit the acquired phenotype of resistance towards pathogenic *V. campbellii* to its (isothermally-reared) subsequent generations. To address this, *Artemia* larvae which were produced ovoviviparously at every generation were challenged with pathogenic *V. campbellii* as described in the methodology section. As shown in **Fig. 3.3A**, the non-exposed T-F1 progeny of F0 *Artemia* that were exposed daily to non-lethal heat shocks exhibited a significantly higher survival when challenged to *V. campbellii* than did the respective progeny of control *Artemia* (C-F1). This relatively higher survival extended to *Vibrio*-challenged T-F2 and T-F3 progenies, whose grandparents and great grandparents, respectively, were exposed to non-lethal heat shocks (**Fig. 3.3B & 3.3C**). In the following experiment, we validated the results of the *Vibrio*-challenge assay by carrying out a common garden experiment, where *Artemia* cysts from all the three (F1 to F3) generations were hatched and the obtained larvae were reared under similar laboratory conditions. Such experiments are useful as it can provide a more mechanistic understanding for the causes of phenotypic variation in the three successive generation progenies in response to heat treatment of the F0 generation. The results showed a similar survival trend as observed for the animals from ovoviviparous reproduction (**Fig. 3.3D- 3.3F**). These data from the common garden experiment reported here, together with the data from the above (generation-specific) challenge tests,

suggested that non-lethal heat-shock treatment of parental *Artemia* also leads to transgenerationally-inherited increase in *Artemia* resistance against the *Vibrio* pathogen.

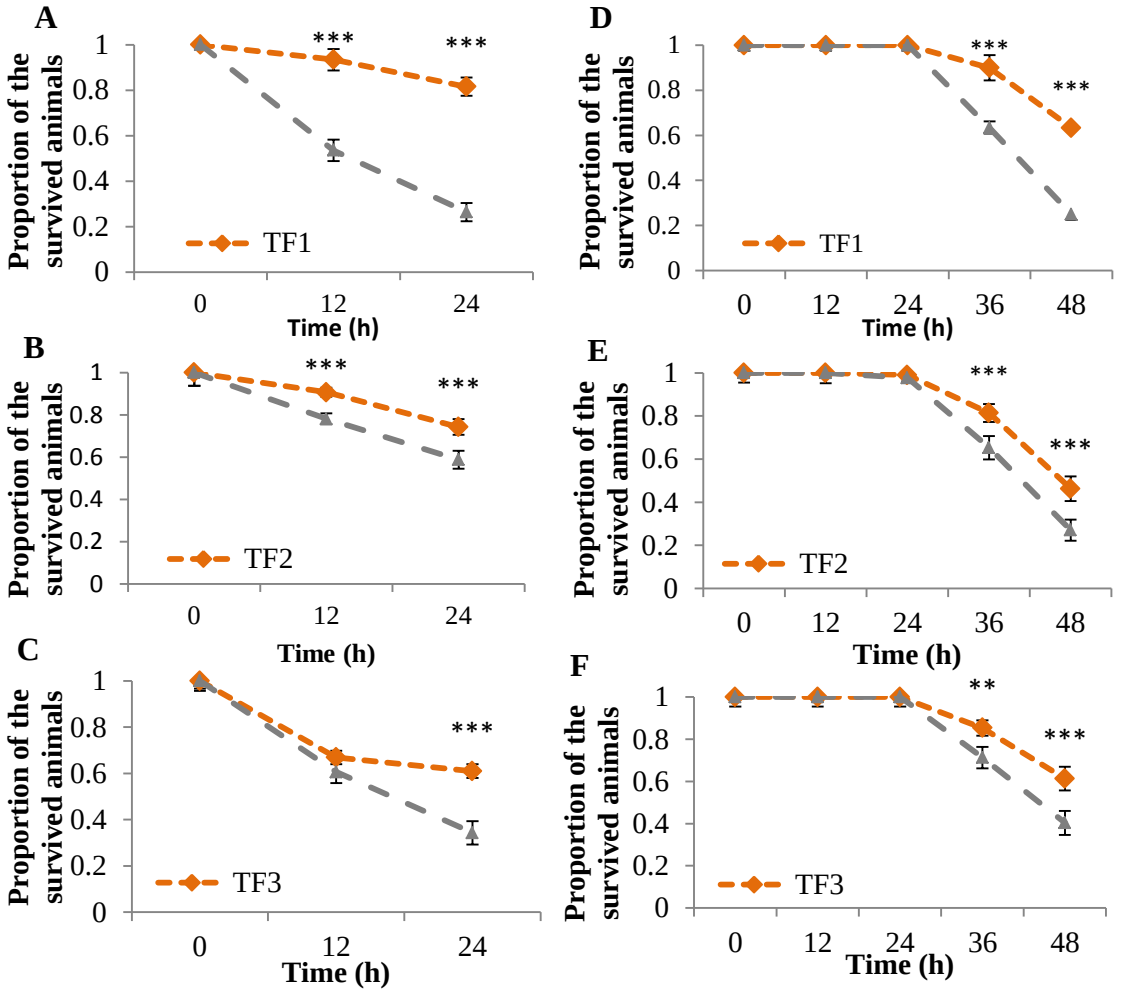

Fig. 3.3: Proportion of the survived *Artemia* larvae challenged with *V. campbellii*. For the treatment (T) and control (C) groups, refer to **Fig. 3.1** for explanation. At every generation, the ovoviviparously produced larvae (at instar II stage) (A, B and C) were challenged with *V. campbellii* at 10^7 cells/mL and survival was scored at every 12 h intervals. Cysts from the generation F1 to F3 were hatched simultaneously, after which the larvae were challenged with *V. campbelii* and survival was scored (common garden test; D, E and F). Values are represented as means ± SE (n = 3). Asterisks represent a significant difference between the control and treatment of the respective generation * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.005$).

Progenies of daily non-lethal heat-shocked ancestors exhibit increased resistance to lethal heat shock in the three subsequent unstressed generations

To substantiate the results of the above challenge studies, we tested how the progenies from all three generations (F1 to F3) of *Artemia* that were exposed to non-lethal heat

shocks, and the respective progenies of control *Artemia*, respond to a heat shock that is lethal for *Artemia* grown at standard temperature. We found that, upon exposure to a typically lethal heat shock (42 °C for 15 min), the survival of the T-F1 progeny of non-lethal heat-shocked F0 parents was significantly higher than the C-F1 progeny of control parents. This result implies that the T-F1 progeny of the non-lethal heat-shocked parent has higher thermo-tolerance than their respective control C-F1 (**Fig. 3.4A**).

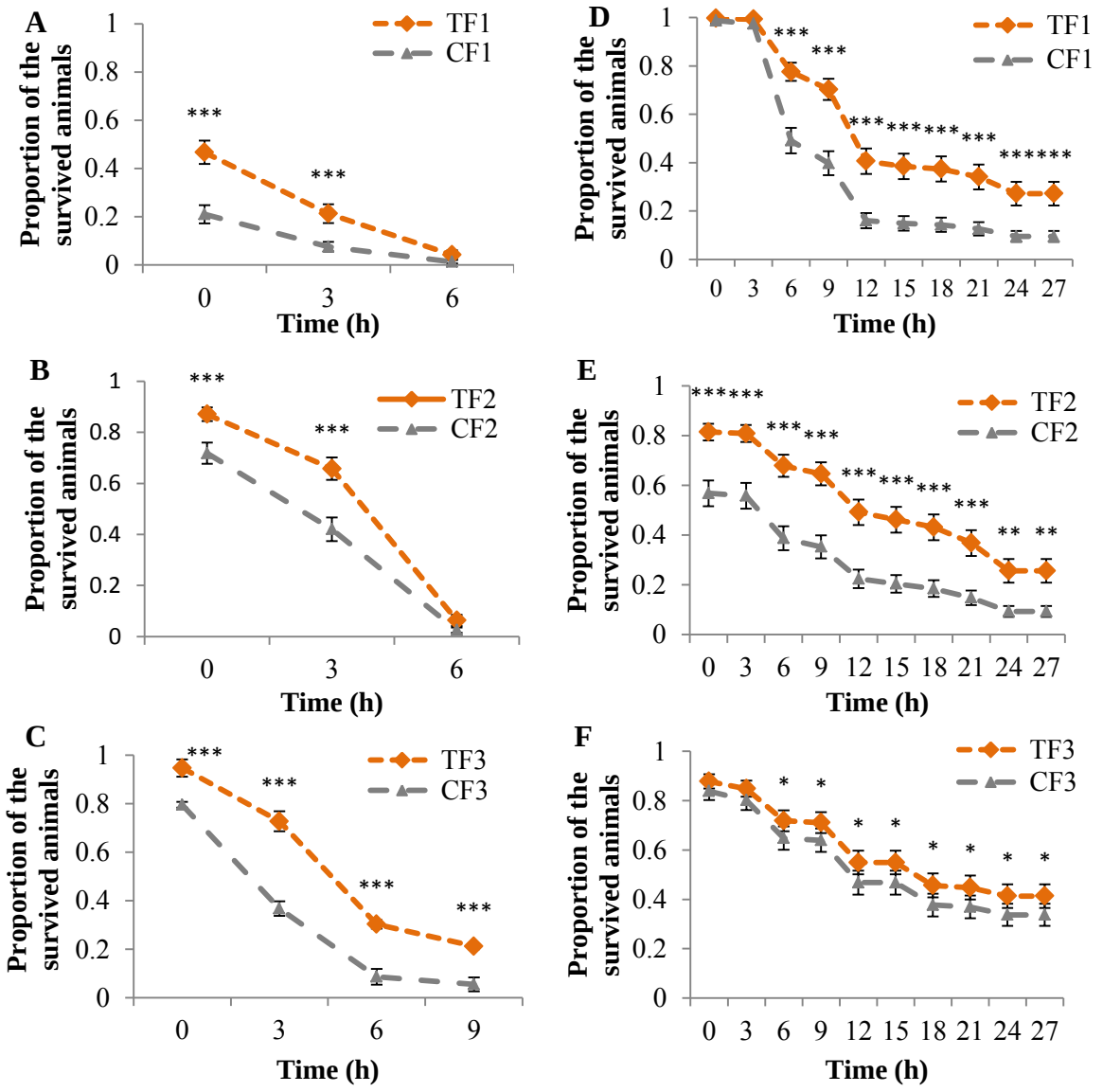

Fig. 3.4: Proportion of the survived *Artemia* larvae challenged with lethal heat shock. For the treatment (T) and control (C) groups, refer to **Fig. 3.1** for explanation. At every generation, the ovoviviparously produced larvae (at instar II stage) (A, B and C) were challenged with lethal heat shock at 42°C for 15 min and survival was scored at 3 h intervals. Cysts from the generation F1 to F3 were hatched simultaneously, after which

the larvae were challenged with *V. campbelii* and survival scored (common garden test; D, E and F). Values are represented as means ± SE (n = 3). Asterisks represent a significant difference between the control and treatment of the respective generation * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.005$).

Interestingly, the increase in thermo-tolerance (with respect to the control groups) was similarly exhibited by T-F2 and T-F3 progenies whose ancestors (T-F0) were treated with non-lethal heat shocks (**Fig. 3.4B & 3.4C**). It is worth noting that the same phenotype was displayed by *Artemia* larvae obtained from ovoviviparous reproduction and by the larvae obtained from cysts in a common garden test (**Fig. 3.4D-3.4F**). These results indicate that, much like the increased Hsp70 production and improved resistance toward pathogenic *V. campbellii*, the non-lethal heat shock-induced stress tolerance has persisted in three successive generations.

Progenies of daily non-lethal heat-shocked ancestors exhibit acetylation of histone H4 and H3 under isothermic conditions

Because previous studies have implicated the critical involvement of histone acetylation in the transcriptional regulation of *hsp70* gene in animals (Chen et al., 2002; Thomson et al., 2004; Zhao et al., 2006; Fritah et al., 2009), we determined if this process is also involved in the observed transgenerationally-inherited upregulation of *Artemia* HSP70 protein. We addressed this by analyzing the acetylation level of histone H4 and H3 (details in Materials and Methods). As shown in **Fig. 3.5 & 3.6**, the level of histone (H4 and H3) acetylation was markedly higher in the non-lethal heat-shocked parental *Artemia* (T-P0) than in the isothermally-grown parental group (C-F0) generation), suggesting that non-lethal heat shocks exert direct or indirect controls on the global level of histone acetylation. Hyperacetylation of histone H4 and H3 was also detected in the T-F1 to T-F3 progenies, whose ancestors were exposed to non-lethal heat shocks. The level of H4 and H3 acetylation appeared to decrease steadily over the generations, a pattern that is reminiscent of the level of HSP70 production described above. Indeed, in the F3 generation there was no significant difference in the level of histone H3 acetylation between the control (C-F3) and treatment (T-F3, **Fig. 3.6**). However, the acetylation of

H3 in the animals with treated ancestors remained relatively higher than the control. An almost similar trend was observed for histone H4 (**Fig. 3.5**).

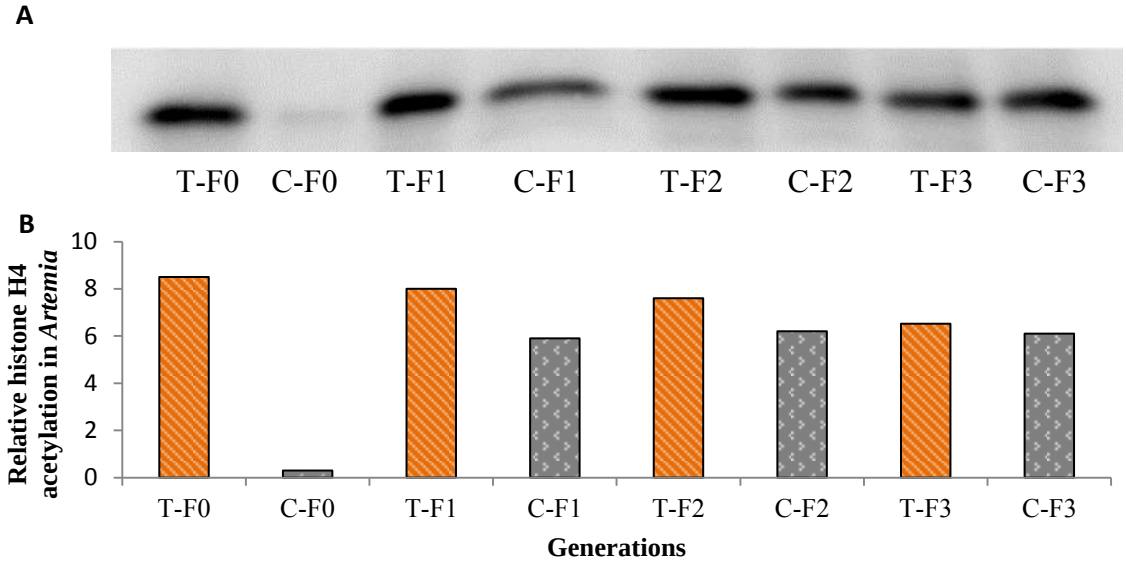

Fig. 3.5: Immunoblot analysis showing acetylation level of histone H4 in the parental *Artemia* and its three successive generation progenies (common garden test). For the treatment (T) and control (C) groups, refer to **Fig. 3.1** for explanation. (A) Total histone extracted from the generation F0 to F3 juveniles was resolved in SDS-PAGE gels and then either stained with Coomassie Biosafe or transferred to polyvinylidene fluoride membrane and probed with antibody that recognizes acetylated lysines at histone H4. *Artemia* histone protein (10 µg) was loaded in each lane. HeLa cells (6 µg) were loaded onto one well to serve as a positive control and for calculating the amount of histone H4 acetylation in the sample. (B) Quantitative analysis of histone H4 in *Artemia* is expressed as ng/µg of histone.

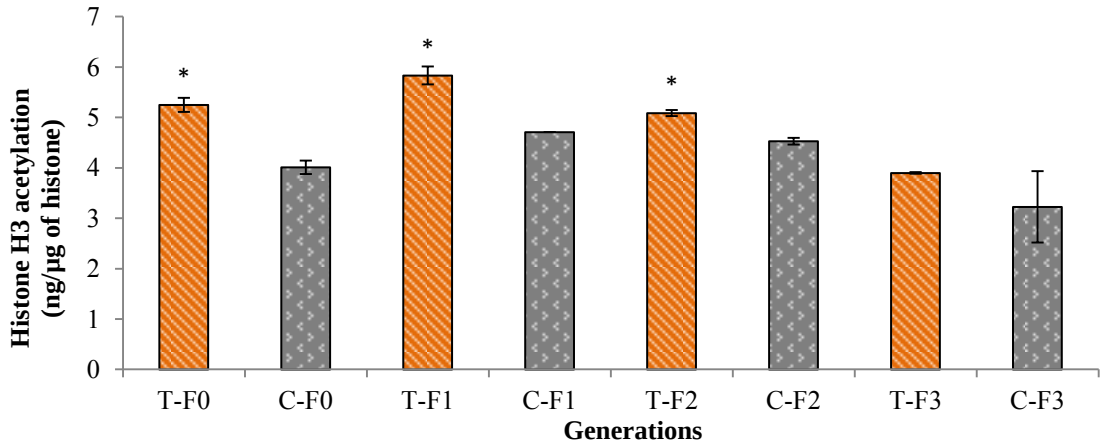

Fig. 3.6: Acetylation level of histone H3 *Artemia* in the parental *Artemia* and their three successive generation progenies (common garden test). For the treatment (T) and control (C) groups, refer to **Fig. 3.1** for explanation. Total histone (5 µg) extracted from

F0 to F3 juveniles was analysed for total histone H3 acetylation using the Episeeker histone H3 total acetylation detection flurometric fast kit. Values are represented as means ± SE (n = 3). Asterisks represent a significant difference between the control and treatment of the respective generation ($P < 0.05$).

These data constitute a powerful argument that acetylation of both H3 and H4 histones contribute, at least to some extent, to the up-regulation of HSP70 production in non-lethal heat-shocked parental *Artemia* and to the inheritance of the increased HSP70 production in successive unstressed generations.

Progenies of daily non-lethal heat-shocked ancestors exhibit changes in global DNA methylation under unstressed conditions

Transgenerational effects in both plants and animals are often associated with alterations in methylation of genomic DNA (Grant-Downton and Dickinson, 2006; Koturbash et al., 2006; Richards, 2008). This prompted us to compare the 5-methylcytosine (5-MeC) content of genomic DNA isolated from treated and control F0 generation as well as from the first three generations progeny by UHPLC-MS/MS analysis. This analysis revealed that the percentage of methylated cytosine in the non-lethal heat-shocked *Artemia* (i.e., T-F0) was approximately 3-fold lower than of non heat-shocked C-F0 group ($P < 0.05$, **Fig. 3.7**).

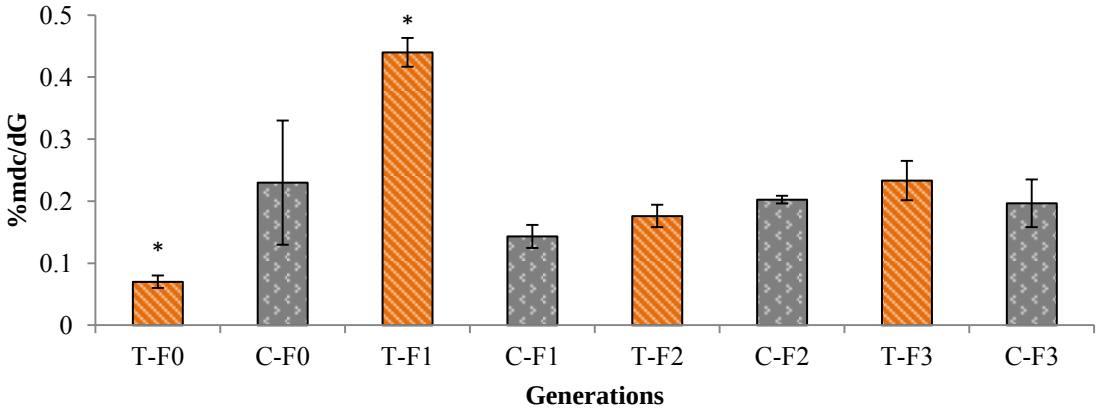

Fig. 3.7: Percentage of methylated cytosine (%mdC/dG) in the parental *Artemia* and its three successive generation progenies (common garden test). For the treatment (T) and control (C) groups, refer to **Fig. 3.1** for explanation. DNA (1 µg) extracted from F0 to F3 juveniles was analysed for global DNA methylation by UHPLC-MS/MS analysis. Values

are represented as means ± SE (n = 3). Asterisks represent a significant difference between the control and treatment of the respective generation ($P < 0.05$).

Interestingly, this response was reversed in the F1 generation, where the T-F1 progenies, reared isothermally, exhibited a significant 3-fold increase in the percentage of cytosine methylation compared to the same generation progeny of control parents (C-F1). In the F2 and F3 generations, the percentage of DNA methylation in the treatment groups (T-F2 and T-F3) dropped down by about 2.5-fold compared to the T-F1 progeny of heat-shocked parent and was statistically indistinguishable from that of the respective C-F2 and C-F3 progenies of the control parental group.

Discussion

The epigenetic inheritance of phenotypic traits across multiple generations is a matter of great interest and of continuing debate (Boyko and Kovalchuk, 2011; De Block and Van Lijsebettens, 2011; Mirouze, and Paszkowski, 2011; Jablonka, 2013). In this study, by using a population produced by apomictic parthenogenetic *Artemia* (i.e. lineages derived from a single mother), we could provide first experimental evidence that environmentally induced phenotypic traits, such as 1) increased HSP70 production, 2) tolerance towards lethal heat shock, and 3) resistance against pathogenic *V. campbellii*, were transmitted to three successive, unexposed generations. Similar phenotypes were observed when this experiment was repeated, indicating the reproducibility of the results. Moreover, we also could demonstrate explicitly that this transmission of the phenotypic traits across three successive generations was associated with changes in the molecular epigenetic marks, such as histone H3 and H4 modification and global DNA methylation.

Studies demonstrating the inheritance of the environmentally responsive phenotypes over multiple generations in (genetically identical) animal or plant models are limited. However there are a few studies on multigenerational transmission of acquired phenotypes. For instance, using a genetically identical *Arabidopsis thaliana* Heynh line plant model, Whittle et al. (2009) demonstrated that plants exposed to a mild heat (30 °C) treatment in the parental and F1 generations exhibited markedly improved fitness (5-fold

increase in seed production per individual) in a later generation (F3). The heat-specific fitness improvements among F3 plants were preserved even after one generation (F2) of reproduction under normal temperature circumstances, which led to the conclusion of an environmentally-induced epigenetic and heritable adaptive phenomenon. Another relevant example of multigenerational inheritance of acquired traits was reported using rats as a model (non-inbred lines) by Benyshek et al. (2006), who demonstrated that impaired glucose metabolism in F1 rats exposed to a nutritional stress during gestation persisted through maternal transmission to the F3 generation. In a further study on a non-inbred line mice model, it was shown that chronic and unpredictable stress in early life of mice altered behavioral response not only in the stressed animals when adult but also in their successive, unstressed generations (up to generation F3) (Franklin, 2010). These examples illustrate that altered phenotypes induced by environmental stress may be transmitted across multiple generations. In our study, we observed the environmentally-induced phenotypic traits in all three successive generations of the parthenogenetic *Artemia* which are having apomictic breeding behavior. Apomixis implies that a parthenogenetic *Artemia* population clone has no other mechanism for genotypic change but mutation, which may induce genetic differentiation (Abatzopoulos et al., 2003). At this point, we cannot exclude the possibility of a genetic (DNA sequence) mutational event (such as, environmentally facilitated single nucleotide polymorphism, SNPs), in the development of observed phenotypes. However, from the available information, it may be suggested that a non-genetic process is also involved in the emergence/inheritance of the observed phenotypic traits or regulation and persistence of epigenetic modifications.

There are ample evidences that expression/production of stress proteins, mainly HSP70, plays important roles in defining the tolerance of organisms to several stressors. For instance, in the invertebrate animal model *Artemia*, we have shown that induction of HSP70 upon exposure to non-lethal heat shock significantly improved the resistance of the animal towards subsequent lethal heat shock or pathogenic *vibrio*s (Sung et al, 2007; Baruah et al., 2012). This improved resistance was due to the fact that HSP70 plays a crucial function as molecular chaperone and is involved in protein biogenesis and protein

homeostasis in the cells or it contributes to the generation of protective immune responses in the host (Clegg et al., 2000; Baruah et al., 2013). Similar increased tolerance to extreme temperatures, both hot and cold, subsequent to the induction of HSP70 by mild heat shock as the initial stressor, has also been demonstrated in other organisms from diverse phyla, including bacteria, coelenterates, molluscs, fish, shrimps, echinoderms and humans (Baruah et al., 2013; Sanders, 1993; Iwama, 1998; Roberts, 2010; Correia, 2013). The positive correlation that we detected in our study between elevated HSP70 levels (as induced by daily non-lethal heat shocks of the T-F0 generation) and increased survival in each generation (T-F1 to T-F3) is in accordance with the above-mentioned studies.

The underlying mechanisms for the inheritance of phenotypes, such as stress tolerance and elevated HSP70 production as observed in our study, remain elusive. However, our analyses suggest that changes in histone acetylation (associated with chromatin relaxation and the initiation of transcription (Fischer et al., 2007) and DNA methylation (mostly known for its gene repression activity (Aguilera et al., 2010) contribute to the emergence and/or multigenerational inheritance of stress tolerance and elevated HSP70 production in *Artemia*. This suggestion is consistent with the known role that these two processes have in shaping epigenetic landscapes, and is compatible with previous observations. As for histone acetylation, for example, a number of studies in animal and plant models have shown that histone acetylation modifications play a key role in epigenetic control and the organism's functional status under stress (Bilichak et al., 2012; Correia et al., 2013) by controlling the functional state of chromatin and gene expression (Myers, 2001; Zhou et al., 2011). For instance, Chen et al. (2002) demonstrated that histone acetylation modification significantly enhanced both the basal and the inducible expression of HSP70 gene in *Drosophila melanogaster*. In another study, Tetievsky and Horowitz (2010) provided evidence that acetylation of histone H4 (but not H3) in HSP70 chromatin is associated with heat shock-induced changes in the HSP70 gene expression. Notably, these authors also showed that histone H4 acetylation is responsible for heat acclimation-mediated cytoprotective memory. In our study, the acetylation states of histone H4 and H3 in the parental generation increased in response to

daily mild heat shocks **(Fig. 3.5 & 3.6).** These modifications propagated up to the F3 generation, in parallel with elevated HSP70 levels and increased stress tolerance in the T-F1 to T-F3 progenies. It has indeed been shown that chromatin modifications can be transmitted to subsequent unstressed generations (Seong et al., 2011). These findings are compatible with a scenario in which acetylated histone H3 and H4 in the T-F1 to T-F3 progenies of treated F0 animals are related in a causal way to HSP70 production. Under this scenario, histone modifications favor prompt binding of transcription factors to heat shock regulatory elements of the HSP70 (or other HSPs) gene, and subsequent rapid activation of the cytoprotective arsenal (Zhao et al., 2006; Bilichak et al., 2012; Tetievsky and Horowitz, 2010).

As for the role that DNA methylation may have in transgenerational epigenetic inheritance, a number of previous studies in animal and plant models have detected an association between alterations in DNA methylation in response to environmental cues and transmission of gene expression patterns and/or adaptation to stress (Anway et al., 2005; Boyko et al., 2010; Verhoeven and van Gurp, 2012). In our study, the methylation patterns of specific genes were not investigated, but global DNA methylation was analyzed as an overall measure of potential stress-induced modifications. Global DNA methylation across four successive generations in response to mild heat shocks of the first generation was highly variable. In fact, at T-F0, we observed a decrease in the global DNA methylation level due to mild heat shocks. This suggests that hypomethylation is needed for upregulation/production of HSPs as observed by a marked increase in the HSP70 level in this T-F0 group. This is in accordance with the results of Gan et al. (2013) who demonstrated that DNA methylation of the HSP70 promoter was negatively associated with the mRNA expression level of HSP70 in the muscle tissue of chicken. On the other hand, the reduced DNA methylation might be a prerequisite for the observed increase histone acetylation (Cedar and Bergman, 2009; Cannuyer et al., 2013). This increased histone acetylation was subsequently transferred to the non-exposed progeny, whereas the DNA methylation was not. By contrast, the T-F1 progenies of heat-shocked parents exhibited a markedly increased global DNA methylation. A similar shift in global

DNA methylation patterns of non-exposed offspring has been observed before in the invertebrate *Daphnia* (Vandegehuchte et al., 2009). This methylation increase can possibly be a compensation effect for the general hypomethylation effect of the heat shock exposure in the T-F0 generations, which might already have been manifested in the F1 germ cells during the F0 exposure. In the T-F2 and T-F3 progenies, the methylation level remained unaffected compared to the C-F2 and C-F3 groups. These generations have never been directly exposed to the heat shocks, even as germ cells.

Our results also showed that there was a decrease in the HSP70 production over the generations. This decrease, however, did not correspond with the phenotypic traits related to increased tolerance against lethal heat shock or against pathogenic *V. campbellii*. In fact, the stress-resistant phenotypes appeared to increase over the generations, with T-F3 progenies being the most robust. This could be explained by the fact that F0-imposed non-lethal heat shocks not only induced HSP70 within the exposed animals but also induced a constellation of stress proteins like HSP27, HSP40, HSP90 (Baruah et al., 2012; Sung et al., 2012) and other stress-resistant genes (Jensen et al., 2008). It is possible that the transgenerational inheritance of stress-resistant traits across generations observed here is mediated by transgenerational inheritance of elevated stress protein expression, including HSP70 and/or other heat-inducible genes potentially involved in stress tolerance. These possibilities need to be addressed by identifying the stress-resistant genes, and their corresponding proteins.

In essence, this study provides strong evidence for the phenomenon that non-lethal heat shocks can induce epigenetic inheritance of phenotypes across three successive generations concomitant with sustained modification of histones H3 and H4. No apparent mechanistic link between global DNA methylation and the acquired phenotypes could be established here. It remains to be determined whether the inheritance of acquired phenotypic traits across generations is linked to DNA methylation, histone acetylation or interplay between them at specific loci, particularly HSP70. Such analysis will provide further mechanistic insight into the observed phenomenon. Overall, these observations

add some intriguing insights to augment our current understanding of transgenerational epigenetic inheritance in animals.

CHAPTER 4

Non-lethal Heat Shock Induces HSP70 and HMGB1 Protein Production Sequentially to Protect *Artemia franciscana* Against *Vibrio campbellii* [1]

Abstract

The high mobility group box 1 (HMGB1) and heat shock protein 70 (HSP70) are DNA and protein chaperones respectively. Apart from being chaperones, these two proteins share many other common characteristics. For instance, they both function as an extracellular signaling molecule and damage associated molecular protein (DAMP) during pathogen encounter, inflammation and various cellular processes. In addition to these, in response to abiotic stressors such as heat stress, the levels of both these proteins greatly alter suggesting their role in cytoprotection. In a previous study on *Artemia* model, it was demonstrated that on exposure to a non-lethal heat stress the protein HSP70 was markedly induced and this contributed to improve resistance of the *Artemia* host against subsequent pathogenic *Vibrio campbellii* challenge. Considering the fact that HMBG1 protein is an intracellular molecular chaperone, and it is released into the extracellular medium upon cellular stress, similar to HSP70, it is possible that the observed protective effects of NLHS is mediated by the induction of HMGB1 in combination with HSP70. Therefore, in this study we aimed to determine the induction of these two proteins in *Artemia* both at the levels of mRNA and protein in response to different factors (i.e. mild heat stress, pathogenic *V. campbellii*, combination of both stimuli). In this study we have shown that in response to mild heat stress, the levels of HSP70 and HMGB1 are elevated sequentially and the significant alterations in the levels of these two proteins were associated with increased protection of *Artemia* against pathogenic *V. campbellii*.

[1] Norouzitallab P, Baruah K, Muthappa DM, Bossier P (2015). Non-lethal heat shock induces HSP70 and HMGB1 protein production sequentially to protect *Artemia franciscana* against *Vibrio campbellii*. Fish & Shellfish Immunology. 42 (2): 395 – 399.

Introduction

Unlike vertebrates that depend on both arms (innate and adaptive) of the immune system, the invertebrates rely only on the innate arsenals to fight against invading pathogens (Roth et al., 2009). Several lines of evidence have suggested that microbial components (such as lipopolysaccharide, proteoglycans, bacterial DNA) and/or signal molecules released from dying cells can readily activate the innate immune system and initiate multiple inflammatory cascades (Erlandsson et al., 2004; Klune et al., 2008). High-mobility group box 1 (HMGB1) and heat shock proteins (HSPs) are well-known mediators of these inflammatory responses (Tsan, 2001). HMGB1 is a highly conserved, ubiquitous, non-histone chromatin-associated protein, which function is to stabilize nucleosome (histone/DNA complex) formation and to act as transcription-factor like protein that regulates gene expressions by bending DNA and promoting access to transcriptional proteins on specific DNA targets (Goodwin et al., 1973; Muller et al., 2001; Lotze and Tracey KJ, 2005; Klune et al., 2008; Tang et al., 2011). Additionally, HMGB1 has recently been reported to induce cytokines and inflammatory responses once secreted to the extracellular environment (Erlandsson et al., 2004). In presence of stressing agent, it acts as a danger signal and inflammatory mediator by passive secretion from necrotic cells (Scaffidi et al., 2010) and/or by an active leakage from immune cells e.g. macrophages, monocytes and dendritic cells (Abraham et al., 2000; Lotze and Tracey, 2005). Like HMGB1 protein, the highly conserved HSPs also play a very comparable role under similar physiological conditions in the organisms (Baruah et al., 2010; Roberts et al., 2010). Under normal biological conditions, HSPs are constitutively produced (intracellularly) and are involved in upholding protein biogenesis and protein homeostasis in the cells (Young et al., 2004; Muchowski and Wacker, 2005). However, under stressful conditions, these proteins are induced and are released either actively or passively into the extracellular environment in order to repair partially denatured proteins, facilitate the degradation of irreversibly denatured proteins and inhibit protein aggregation, thus protecting cells from harmful environmental stresses (Mayer et al., 2005; Wang et al., 2009). Besides these, extracellular HSPs are also implicated in

eliciting immune responses against many diseases as demonstrated in a wide variety of experimental models (Tsan and Gao, 2009; Chen and Cao, 2010; Baruah et al., 2013). These proteins range in size from 27 to 110 kDa and are categorized into five main families according to their molecular mass: HSP100, HSP90, HSP70, HSP60 and small HSPs (Almeida, 2011). Among the different HSP families, the HSP70 family molecules, such as the constitutive HSP70 (HSC70 or HSP73) and the stress-inducible HSP70 (HSP70 or HSP72), are the most well-characterized HSPs (Chen et al., 2010).

The proteins HMGB1 and HSPs share many common characteristics. For instance, they function as molecular chaperone for DNA and protein, respectively, both function as an extracellular signaling molecule and damage associated molecular protein (DAMP) during inflammation and various cellular processes (see review Tang et al., 2011; Lu et al., 2014). In presence of pathogenic biotic stressors, extracellular HMGB1 and HSPs participate in the activation of cell surface innate immune receptors, typically Toll-like receptors (TLRs), thereby affecting many aspects of host's immune responses (Lotze et al., 2005; Harris et al., 2012). In addition to these, abiotic stressors such as heat stress can also readily alter the levels of both these proteins (Tang et al., 2005; Tang et al., 2011).

In our previous study, we have shown that exposure to a non-lethal heat stress (NLHS) at 37 °C for 30 min followed by 6 h recovery period induced HSP70 production within the host *Artemia fransciscana* nauplii and this induced HSP70 contributed to improve resistance of the host against subsequent *Vibrio* challenges (Sung et al., 2007). Considering the fact that HMBG1 protein is an intracellular molecular chaperone, and it is released into the extracellular medium upon cellular stress or activation, similar to HSP70 (Tsan et al., 2011), it is possible that the observed protective effects of NLHS is mediated by the induction of HMGB1 in combination with HSP70. In this first study, using the gnotobiotic *Artemia* model organism (GART) system, we investigated whether these two molecular chaperones are the effector molecules in mediating downstream protection to *Artemia* against pathogenic *Vibrio campbellii.* We used the GART system to conduct this study since in this system *Artemia* can be cultured under germ-free environment, and a controlled species and population of micro-organism can be added.

This system is a crucial tool for such studies because it allows eliminating the interference of the microbiota that are naturally present in any type of aquatic environment and furthermore facilitates the interpretation of the results in terms of a cause effect relationship (Marques et al., 2004; Baruah et al., 2014).

Materials and methods

The gnotobiotic system was developed by hatching of *Artemia* cysts axenically following decapsulation and hatching procedures as described previously (Baruah et al., 2009). Briefly, 12 g of *Artemia* cysts originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were hydrated in 89 mL of sterile distilled water for 1 h. Sterile cysts and nauplii were obtained after decapsulation via using 3.3 mL NaOH (32%) and 50 mL NaOCl (50%). The reaction was stopped after 2 min by adding 50 mL $Na_2S_2O_3$ (10 g/L). Thereafter the decapsulated cysts were washed with sterile artificial seawater (35 g/L, Aquarium Systems, Sarrebourg, France) and then suspended in 1 L glass bottles containing sterile artificial seawater, and then incubated at 28 °C for 28 h with constant illumination of approximately 27 $\mu E/m^2$.sec for hatching. All the manipulations were performed under a laminar flow hood and all tools were autoclaved at 121 °C for 20 min in order to maintain sterility of the cysts and nauplii. After 28 h of incubation, the axenicity of the hatched *Artemia* nauplii was verified both by spread plating (100 mL) and by adding (500 µL) hatching water on Marine Agar and in Marine Broth (Difco, Detroit, USA), respectively followed by incubation at 28 °C for 5 days (Baruah et al., 2009). Experiments starting with non-sterile nauplii were discarded.

Swimming nauplii at instar II stage were collected, counted volumetrically and then distributed in 4 groups, each with 3 replicates. Each group was maintained in 1 L glass bottle containing sterile seawater and placed in rectangular tank containing water maintained at 28 °C using a thermostatic heater with constant illumination (approximately 27 $\mu M/m^2$.sec) and aeration. Two groups were given non-lethal heat shock treatment by exposing the nauplii to a temperature of 37 °C for 30 min following the procedure

described previously (Sung et al., 2007). The other two groups were maintained isothermally at 28 °C. One group, each from the heat shocked and non-heat shock, were challenged with *Vibrio campbellii* at 10^7 cells/mL for 12 h (**Chapter 3**). Samples containing a group of live nauplii weighing in total 0.1 g were harvested from each group at 6 and 12 h post challenge, rinsed in sterile distilled water, immediately frozen in liquid nitrogen and preserved at -80 °C for further analysis.

Total RNA was extracted from each *Artemia* sample using the RNA extraction kit (Promega, Belgium). First strand cDNA was synthesized from 1 µg total RNA using the RevertAid™ H minus First strand cDNA synthesis kit (Fermentas Gmbh, Germany) following the manufacturer's guidelines. The expression of *hsp70* and *hmgb1* genes in the nauplii was analyzed by qRT-PCR using a following pair of primers (*hsp70*: forward - cgataaaggccgtctctcca, reverse-cagcttcaggtaacttgtccttg; *hmgb1*: forward-ggatgaaagcaaaccccgtg, reverse - gtgctcttctctgcaagtctg). The primers for *Artemia hsp70* gene were designed previously by Baruah et al. (2010) based on the *Artemia* HSP70 cDNA sequence provided by Dr. Thomas H. MacRae. The primers for the *hmgb1* gene were designed based on the region of the *Artemia hmgb1* gene (information available through an internally available *Artemia* draft genome) that is highly conserved with human *hmgb1* gene (accession No. LN609733). The qRT-PCR amplifications were carried out in a total volume of 25 µL, containing 9.8 µL of nuclease free water, 0.4 µL of each primer, 12.5 µL of Maxima SYBR Green qPCR Master mix (Fermentas, Cambridgeshire) and 2 µL of cDNA template. The qRT-PCR was performed in a One Step qRT-PCR instrument (Applied Biosystems) using a four-step amplification protocol: initial denaturation (10 min at 95 ^{o}C); 40 cycles of amplification and quantification (15 s at 95 ^{o}C, 30 s at 60 ^{o}C, and 30 s at 72 °C); melting curve (55-95 ^{o}C with a heating rate of 0.10 °C/s and a continuous fluorescence measurement) and cooling (4 °C). The *β-actin* gene was used as a reference gene for standardizing the expression of target genes. Master mixes were prepared in duplicate for each sample and qRT-PCR for target and reference genes was performed. Relative quantification of target gene transcripts with a chosen

reference gene transcript was done following the Pfaffl method (Pfaffl, 2002; Baruah et al., 2011).

Artemia samples were homogenized in cold buffer K, centrifuged at 2200 x *g* for 1 min at 4 °C and the supernatant was collected. Protein was quantified by the Bradford method (Bradford, 1976) using bovine serum albumin as standard. Loading buffer was added to the supernatant samples, vortexed, heated at 95 °C for 5 min and electrophoresed in 10% and 4-20% SDS-PAGE gel for HSP70 and HMGB1, respectively, with each lane receiving 10 µg of protein. HeLa (heat shocked) cells (Enzo Life Sciences, USA) (6 µg) were loaded on to one well to serve as a positive control and for calculating the amount of target proteins in the sample. Proteins were then electrotransferred to polyvinylidene fluoride membranes (BioRad Immun-Blot™ PVDF) for antibody probing. The membranes were incubated with blocking buffer [50 ml of 1x phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 60 min at room temperature followed by with primary antibodies for HSP70, which recognizes both constitutive and inducible Hsp70 (1:5000; Affinity BioReagents Inc., Golden, CO) (Bradford, 1976) and HMGB1 (1:2500; Affinity Abcam, ab18256, United Kingdom). The membranes were then incubated with horseradish peroxidase conjugated donkey anti-mouse IgG (1: 2500; Affinity BioReagents Inc., Golden, CO) and horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibodies (1:10000; Gentaur BVBA, Belgium) for HSP70 and HMGB1, respectively. The membranes were then treated with enhanced chemiluminescence reagent (GE healthcare, UK) and the signals were detected by a ChemiDoc MP Imaging System (Biorad, Belgium). The relative signal intensity was quantified by densitometry with Biorad Image Lab™ Software version 4.1.

The HSP70 and HMGB1 protein data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests using the statistical software Statistical Package for the Social Sciences version 20.0. *P* values ≤ 0.05 were considered significant. Results for *hsp70* and *hmgb1* mRNA quantification are presented as fold expression relative to *Artemia* actin. The expression level in the control group was regarded as 1.000 and thereby the expression ratio of the treatments was expressed in

relation to the control. Significant differences in expression between control and treatments were analyzed by Pfaffl method (Pfaffl, 2002).

Results and discussion

In our previous studies, HSP70 was investigated for its role in controlling bacterial disease in (shrimp) aquaculture (Sung et al., 2007; Baruah et al., 2012). For that HSP70 protein was induced within the aquaculture model organism *Artemia* by exposing the animal to a classical stress inducer i.e. a non-lethal heat shock at 37 °C for 30 min followed by 6 h recovery period. In those studies, HSP70 protein was analysed immediately following the end of the recovery period, and this phenomenon of increased HSP70 production level was shown to be associated with conferring protection to *Artemia* against subsequent *Vibrio* attacks (Sung et al., 2007). The current study is an extension of our earlier studies (Sung et al., 2007; Baruah et al., 2012). However, here we analysed HSP70 and HMGB1, both at the transcriptional and translational levels, in *Artemia* exposed to a non-lethal heat shock at 37 °C for 30 min (but without a recovery period) and immediately challenged with *V. campbellii* for an indicated period, with the aim to determine if besides HSP70, HMGB1 protein is also induced in response to non-lethal heat shock, and whether these two proteins are involved in conferring protection to *Vibrio*-challenged *Artemia*. Our results showed that at 6 h post challenge, non-lethal heat shock treatment caused a significant effect on the expression of *hsp70* gene in *Artemia* (**Fig. 4.1**). In fact, due to non-lethal heat shock treatment, the hsp70 expression level in the unchallenged (NLHS group) and *Vibrio*-challenged (NLHS+VC group) *Artemia* increased by respectively 3.1-fold and 2.7-fold relative to the corresponding control. Exposure of *Artemia* to *V. campbellii* (VC group) for 6 h also caused a significant increase (6.3-fold to the control, $P < 0.05$) in the expression level of hsp70 mRNA. However, at 12 h post challenge, the hsp70 mRNA level did not differ significantly among the different groups ($P > 0.05$, Fig 1). Having observed these, we next verified if the transcribed *hsp70* in *Artemia* due to different treatments was translated to functional protein to exert its functions. To this end, we carried out Western blot analysis and the

results revealed that non-lethal heat shock treatment significantly increased HSP70 production in the *Artemia* as compared to the untreated control group at 6 h post challenge (**Fig. 4.2A & 4.2C**).

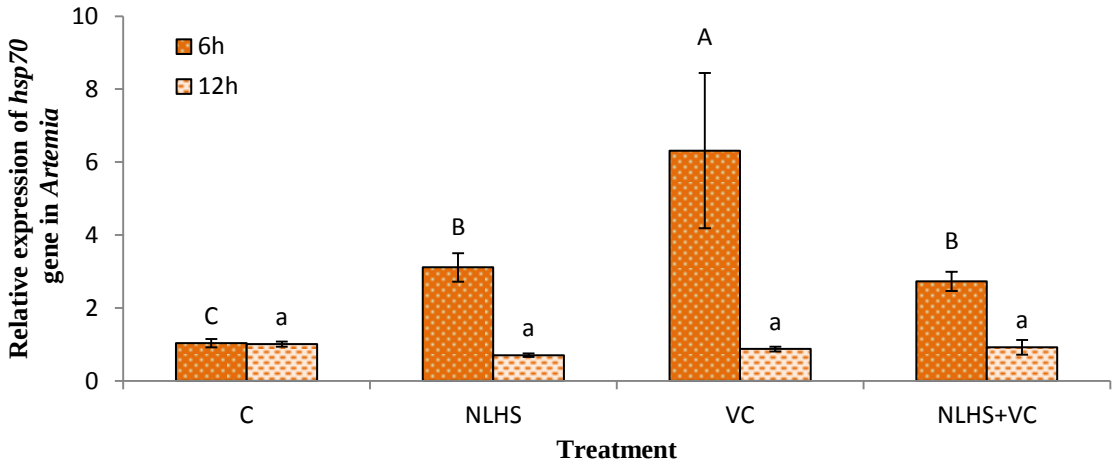

Fig. 4.1: Expression of *hsp70* gene in *Artemia* nauplii. The nauplii were exposed to non-lethal heat shock (NLHS) treatment at indicated conditions. The nauplii were then either challenged for 12 h with *V. campbellii* (NLHS+VC) or not. Untreated *Artemia* challenged with *V. campbellii* (VC) or not (C) served as controls. Samples were collected for *hsp70* gene expression at 6 and 12 h post challenge. The expression of *hsp70* mRNA in the control (C) group was regarded as 1. Results, which are the mean of 3 replicates, are presented relative to Artemia actin gene expression, according to the equation of Pfaffl et al. (2002). Bars indicate standard error. Significant differences among the groups at corresponding time points are indicated by different letters (capital and small letters for 6 and 12 h, respectively; $P < 0.05$).

However, at 6 h post challenge, the HSP70 protein level in the untreated *Artemia* challenged with *V. campbellii* (VC group) was significantly reduced (**Fig. 4.2A & 4.2C**), and this reduction in the HSP70 production level occurred in spite of the increase in the expression level of *hsp70* gene in this group (**Fig. 4.1**). By inducing HSP70, organisms temporally and spatially respond swiftly to an ever-changing array of environmental conditions (for review, see Roberts et al., 2010). For instance, it has been unequivocally demonstrated that synthesized HSP70 protein in animals contributes to the generation of protective (immune) responses against subsequent bacterial attacks (Wang et al., 2009; Baruah et al., 2010; Wheeler et al., 2011; Baruah et al., 2012). However, there are also evidences suggesting that to survive the host immune response, bacteria respond to the elevated signaling molecules (such as HSP70 mRNA) by targeting the host mRNA

translation machineries and thereby inhibiting the synthesis of functional proteins involved in cellular homeostasis and cellular survival (Chakrabarti et al., 2012; Mohr and Sonenberg, 2012). From this compendium of evidences, we can hypothesize that the reduced HSP70 production in the challenged *Artemia* (VC group) could be due to the interference of the pathogenic *V. campbellii* with the HSP70 translation machineries of the host. Our results also showed that the HSP70 production level in heat-shocked group challenged with *V. campbellii* (NLHS+VC group) was significantly reduced as compared to the NLHS group (**Fig. 4.2A & 4.2C**; $P < 0.05$), however, not to a level that it differed significantly from that in the control. This indicates that NLHS exposure might have minimized the functional activity of *V. campbellii* in interfering with the host HSP70 translation machineries, a hypothesis that needs further verification.

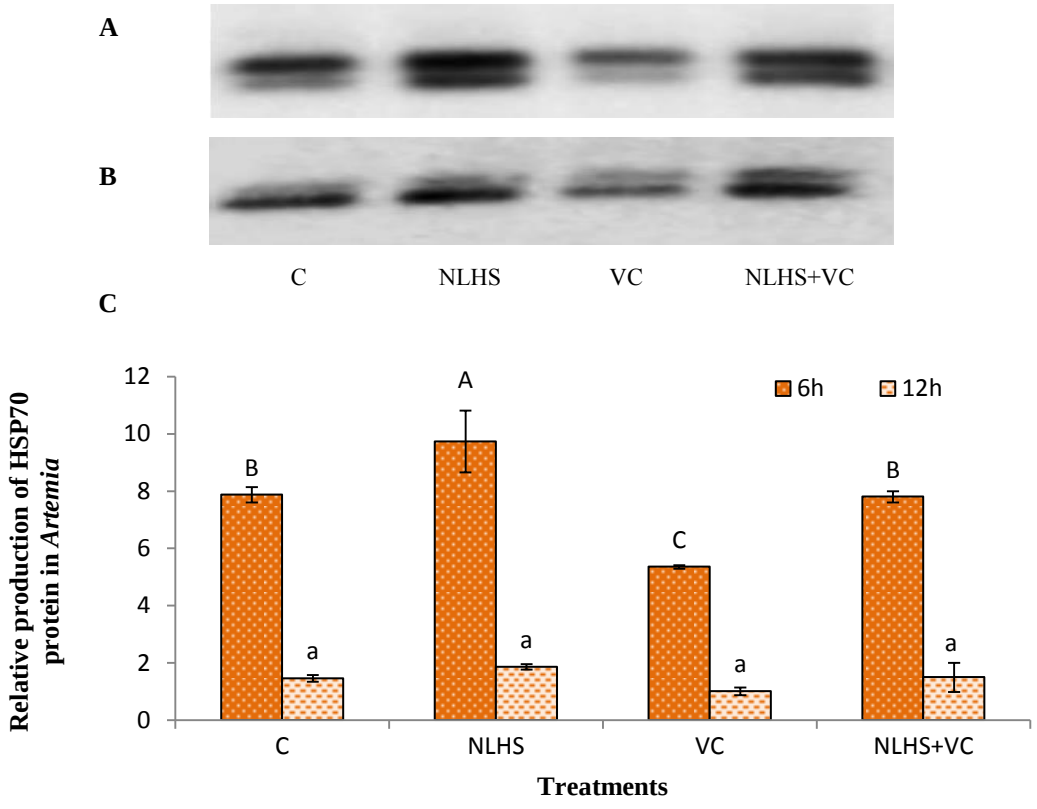

Fig. 4.2: Production of HSP70 protein in *Artemia* nauplii. For the control and treatment groups, refer to **Fig. 4.1** for explanation. Extracted protein from *Artemia* samples collected at (A) 6 h and (B) 12 h post challenge was resolved in SDS-PAGE gel, transferred to polyvinylidene fluoride membranes and then probed with anti-HSP70 antibody. *Artemia* protein (10 µg) was loaded in each lane. HeLa (heat shocked) cells (6 µg) were loaded on to one well to serve as a positive technical control and for calculating

the relative amount of HSP70 in the samples. (C) Quantitative analysis of HSP70 in *Artemia* (expressed relative to the amount in HeLa cells). Bars indicate standard error of 3 replicates. Significant differences among the groups at corresponding time points are indicated by different letters (capital and small letters for 6 and 12 h, respectively; $P < 0.05$). Dual bands appeared in **Fig. 4.2A** and 2B. The upper and lower bands are predicted to represent constitutive HSP70 (HSC70 or HSP73) and the stress-inducible HSP70 (HSP70 or HSP72).

At 12 h post challenge, similar to what was observed for *hsp70* gene expression; the HSP70 protein level did not differ significantly among the different groups (**Fig. 4.2B & 4.2C**). Despite the absence of increased (or limited) production of HSP70 in NLHS-*Artemia* challenged with *V. campbellii* at the indicated time points, we observed a significant increase in the survival of NLHS-*Artemia* challenged with *V. campbellii* as compared to the untreated *Artemia* challenged with *V. campbellii* (data not shown, see Sung et al., 2007; Baruah et al., 2012). In this study, HSP70 was analyzed at about 6 h and 12 h post exposures to NLHS/*V. campbellii*. It is possible that early production (prior to 6 h) of HSP70 and/or other members of HSP family like HSP60, HSP90 protein in response to NLHS might have contributed to the increased survival of *Vibrio*-challenged *Artemia*. Also the observed survival could be due to the elevated levels of HMGB1 molecule instigated by increased levels of HSP70.

HMGB1 is a nuclear protein, which functions as a nucleosome stabilizer and a regulator of transcription (Lotze and Tracey, 2005). But the activity of HMGB1 is not solely mediated by its ability to bind to DNA. Indeed, recent evidences suggested that in response to stimuli such as infection and injury (oxidative stress) this DNA chaperone can get released into the extracellular environment and can instigate the host immune system to mount a nonspecific biological responses at the site of infection or injury (Tian et al., 2007; Yana et al., 2009). Since a link exists between HMGB1 induction and oxidative stress and downstream survival of an organism (Tang et al., 2011), we further analyzed the induction of HMGB1 both at the gene and protein levels in *Artemia* exposed to different factors. As shown in **Fig. 4.3**, there was no significant difference in the expression level of *hmgb1* gene among the different groups at any of the time points tested. However, a significant change in the level of HMGB1 protein in response to the

NLHS and/or *V.campbellii* was observed (**Fig. 4.4**). At 6 h post challenge, the HMGB1 protein level in *Artemia* exposed to NLHS and *V. campbellii* decreased by 1.4- and 1.6-fold, respectively compared to the control ($P < 0.05$, **Fig. 4.4 A & 4.4C**). However, in the NLHS-*Artemia* immediately challenged with *V. campbellii* (NLHS+VC group), the HMGB1 protein level was comparable to that of the control group.

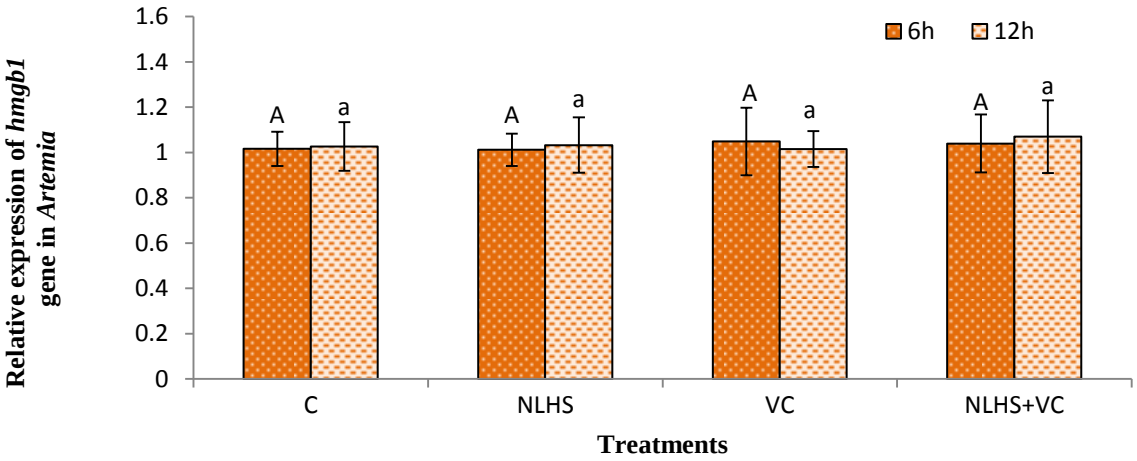

Fig. 4.3: Expression of hmgb1 gene in *Artemia* nauplii. For the control and treatment groups, refer to **Fig. 4.1** for explanation. Samples were collected for *hsp70* gene expression at 6 and 12 h post challenge. The expression of *hmgb1* mRNA in the control (C) group was regarded as 1. Results, which are the mean of 3 replicates, are presented relative to *Artemia* actin gene expression, according to the equation of Pfaffl et al. (2002). Bars indicate standard error. Significant differences among the groups at corresponding time points are indicated by different letters (capital and small letters for 6 and 12 h, respectively; $P < 0.05$).

At 12 h post challenge, the HMGB1 protein level in untreated *Artemia* challenged with *V. campbellii* (VC group) remained altered compared to the control group ($P > 0.05$, **Fig. 4.4B & 4.4C**). However, in response to NLHS exposure, there was a significant increase in the production of HMGB1 protein in both the unchallenged (NLHS group, 1.3-fold) and challenged (NLHS+VC group, 1.4-fold) *Artemia*. These phenomena, interestingly, corresponded well with increased survival of *Artemia* in these groups (data not shown; see Sung et al., 2007). Taken together, these results indicate that in parallel to HSP70, HMGB1 protein is also induced in *Artemia* in response to exposure to NLHS and these two molecular chaperones (possibly) through an unexplored cascade of biochemical and immunological reactions might have contributed to the protection of *Artemia* against

V. campbellii. This finding of our study corroborates another report that is pointing towards a critical role of HMGB1 protein in conferring protection to mice against LPS–induced endotoxemia and bacterial infection by *Listeria monocytogenes* (Yana et al., 2009).

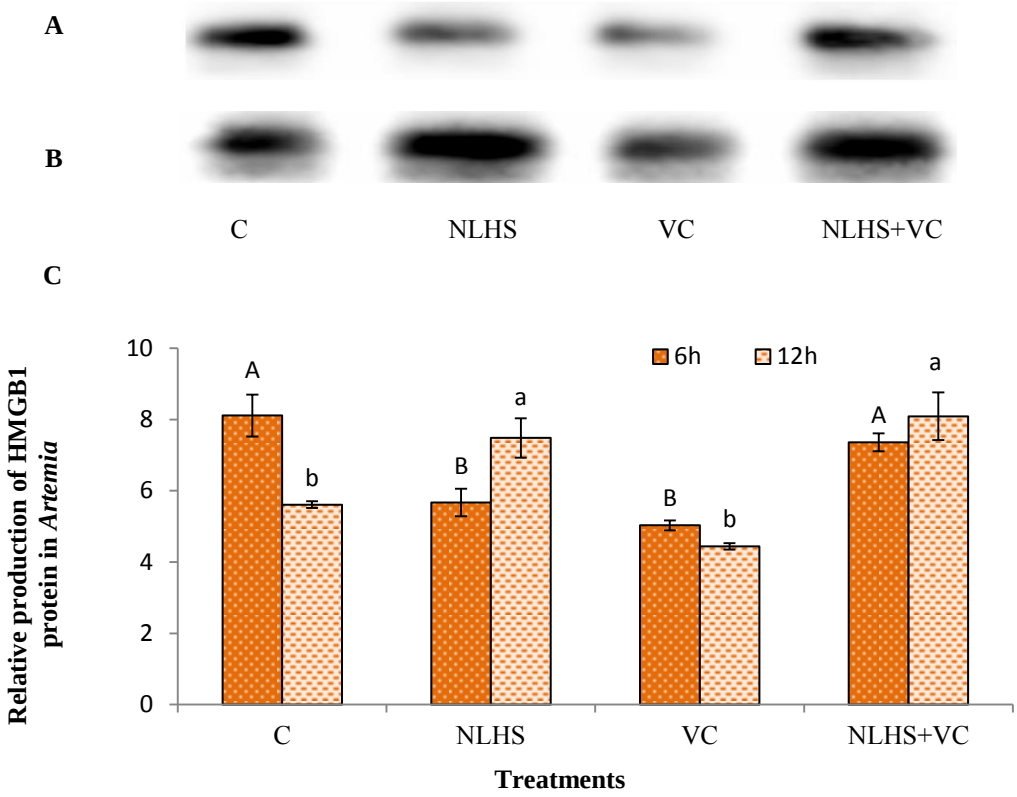

Fig. 4.4: Production of HMGB1 protein in *Artemia* nauplii. For the control and treatment groups, refer to Fig. 1 for explanation. Extracted protein from *Artemia* samples collected at (A) 6 h and (B) 12 h post challenge was resolved in SDS-PAGE gel, transferred to polyvinylidene fluoride membranes and then probed with anti-HMGB1 antibody. *Artemia* protein (10 µg) was loaded in each lane. HeLa (heat shocked) cells (6 µg) were loaded on to one well to serve as a positive technical control and for calculating the relative amount of HMGB1 in the samples. (C) Quantitative analysis of HMGB1 in *Artemia* (expressed relative to the amount in HeLa cells). Bars indicate standard error of 3 replicates. Significant differences among the groups at corresponding time points are indicated by different letters (capital and small letters for 6 and 12 h, respectively; $P < 0.05$).

A striking observation that was made in this study was that the induction pattern of HSP70 and HMGB1 proteins in response to NLHS was inversely related, suggesting that these two molecular proteins may be induced sequentially, but necessarily causally, to execute their cytoprotective and immunoregulatory functions. The mechanisms

underlying the regulation of active HMGB1 release, association with HSP70 are complex and remain elusive, and therefore need further verification.

In conclusion, this study provides strong *in vivo* evidences that the levels of HSP70 and HMGB1 in a gnotobiotically-grown *Artemia* are elevated sequentially, rather than simultaneously, in response to mild heat stress. These significant alterations in the level of these two proteins were associated with increased protection of *Artemia* against *V. campbellii*. In view of the fact that HSP70 and HMGB1 proteins showed anti-bacterial functions, further studies are warranted to unravel the molecular mechanisms that underlie the protective effect of these two proteins against bacterial infection.

PART 3

Epigenetic Control of Innate Immune System in *Artemia*

It is not enough to discover and prove a useful truth that was previously unknown, but it is also necessary to propagate it and get it recognized.

- Jean-Baptiste Lamarck (1744 -1829)

CHAPTER 5

Multigenerational Immune Priming in an Invertebrate Parthenogenetic *Artemia* to Pathogenic *Vibrio campbellii* [1]

Abstract

Previously, it was widely accepted that invertebrates rely only on the innate arm of defense system and they fail to show specificity and memory in their immune responses. Recent reports however, have challenged this view by demonstrating that invertebrate can exhibit memory, specificity and/or maternal transfer of immunocompetence like that of vertebrate adaptive immune system. This form of immunity in invertebrates is termed 'immune priming', which is broadly defined as increased protection to a pathogen following previous exposure to the same pathogen or an immune elicitor. A few recent studies have indicated that similar to vertebrate adaptive immunity, the immune priming phenomenon in invertebrates can be transgenerational i.e., the protective/immune responses are passed from parents to offspring. However, those studies were carried out only up to the first-generation progeny. In addition in many of those studies the observed immune priming phenotype could be due to selection rather than paternal/maternal transfer of immune-priming traits. Here, we used the well-developed host-pathogen laboratory model system, apomictic parthenogenetic *Artemia* (clonal population) and its pathogenic bacteria *Vibrio campbellii* to address the phenomenon of multigenerational immune priming in invertebrate. By using clonal population, we reduced the chance of selection. Our results demonstrated that immune-challenging *Artemia* by specific bacteria, bestow their next generation progenies with increased resistance against the same and this acquired strain-specific immunity is further transmissible to three successive generation progenies.

[1] Norouzitallab P, Biswas P, Baruah K, Bossier P (2015). Multigenerational immune priming in an invertebrate parthenogenetic *Artemia* to a pathogenic *Vibrio campbellii.* Fish & Shellfish Immunology. 42(2): 426–429.

Introduction

Invertebrates were traditionally thought to rely on the innate arm of the defense system to fight against pathogens (Hoffmann and Reichhart, 2006; Baruah et al., 2014), and adaptivity or memory of immune responses were previously considered the hallmark of a highly evolved immune system, only present in vertebrates (Litman, 2014). However, there are now evidences indicating that invertebrates have immunological specificity and memory of a kind that is a functional equivalent of adaptive immunity in vertebrates (Little et al., 2005). These claims for adaptive-like immunity in invertebrates were based largely on a few (phenomenological) observations that previous exposure to pathogen and/or immune elicitor (like lipopolysaccharide, heat shock protein, glucan) has a positive impact on the immune system components (Sadd et al., 2005; Moret, 2006; Pham et al., 2007; Tidbury et al., 2011) and on the resistance of the animals against subsequent microbial attacks (Roth et al., 2010; Sadd and Schmid-Hempel, 2011). This form of memory in an invertebrate is termed 'immune priming', which is broadly defined as increased protection to a pathogen following previous exposure to a pathogen or an immune elicitor. A few recent studies have indicated that similar to vertebrate adaptive immunity, the immune priming phenomenon in invertebrates is transgenerational i.e., the protective/immune responses are passed from parents to offspring (Sadd et al., 2005; Tidbury et al., 2011; Freitak, 2014). For example, in the insect *Trichoplusia ni*, offspring from mothers that had been raised on a bacteria-rich diet had an increased immune response in terms of immune enzyme activity, and the expression of immune-related genes (Freitak, 2012). In addition, in the red flour beetle, *Tribolium castaneum*, it was shown that after parental exposure to heat-killed bacteria, transgenerational immune priming occurs through fathers as well as mothers (Roth et al., 2010). However, in most of the previous studies on transgenerational immune priming in invertebrates, assessments of the maternally/paternally-transferred acquired immune responses or disease-resistant traits were carried out only in the first-generation progeny. If there is immune memory in invertebrates, it can be expected that the acquired memory-like immune responses or disease-resistant traits in the parental generation are transmitted across subsequent

generations. However, at present, little evidence appears in the literature that unequivocally demonstrates the transfer/persistence of (immune) priming effects across subsequent generations.

Here, we used the well-developed host-pathogen laboratory model system, apomictic parthenogenetic *Artemia* (an aquatic invertebrate) and its pathogenic bacteria *Vibrio campbellii* strain LMG21363 to address the phenomenon of multigenerational immune priming in invertebrate. The pathogen *V. campbellii* was selected because it is an opportunistic bacterium that causes significant mortalities in the farmed aquatic animals, including *Artemia* (Baruah et al., 2014). In particular, we immune challenged a population of apomictic parthenogenetic *Artemia* (obtained from a single female) by exposing the population at early life stages to *V. campbellii* and examined the resistance of the three successive generation progenies (none of them were immune challenged) by challenging with the same strain of bacteria. We demonstrated for the first time in a crustacean/invertebrate that immune-challenged *Artemia* by specific bacteria bestow their next generation progenies with increased resistance against the same strain of bacteria and this acquired strain-specific immunity is further transmissible to successive generation progenies.

Materials and methods

A population of apomictic parthenogenetic *Artemia* was obtained ovoviviparously from a single tetraploid female of Megalon Embolon saltworks in Greece (Baxevanis and Abatzopoulos, 2004). The animals were grown till adult in 35 g/L artificial seawater under controlled laboratory conditions as described previously (**Chapter 3**). Throughout the culture period, the animals were fed *ad libitum* everyday with live green microalgae *Tetraselmis suecica* obtained from the Culture Collection of Algae and Protozoa (CCAP) Department (Dunstaffnage Marine Laboratory, Oban, UK). Under non-optimal environmental conditions, such as high salinity or low oxygen, parthenogenetic *Artemia* switch from an ovoviviparous to an oviparous mode of reproduction. To induce the *Artemia* to produce cysts, the salinity of the culture water was gradually increased from

35 to 80 g/L over a period of 10 days. Deposited cysts enter in a state of diapause and are not ready for hatching (Nambu et al., 2008). To terminate cyst diapause, the collected cysts were dehydrated in a saturated NaCl brine solution and exposed to a temperature of -20 °C for 3 months, and then stored as activated cysts at 4 °C until use. For the experiment, the cysts were hatched under axenic conditions (to avoid or minimize the initial load of bacteria in the culture condition) via decapsulation using NaOH (32%) and NaOCl (50%) as described previously (**Chapter 4**). The sterile decapsulated cysts were transferred to 1 L glass bottles containing 35 g/L of sterile artificial seawater. Following incubation at 28 °C under constant illumination for 48 h (or 2 days posthatching, dph), the emerged instar II larvae (stage at which mouth is open for ingestion of food) were used for experimental treatments.

A group of 800 instar II nauplii (2 dph) was distributed in two groups (treatment and control), each with 3 replicates. Each group was maintained in a 1 L glass bottle containing sterile artificial seawater (35 g/L), maintained at 28 °C under constant illumination (approximately 27 μmol/m^2. s) and aeration. The treatment groups (on 6 dph) were exposed to 10^7 cells/mL concentration of *V. campbellii* strain LMG21363 for a period of 3 days. On 10 dph, the nauplii from the bottles were collected over a sieve (250 μm), rinsed several times with sterile seawater to wash away the bacteria associated with the nauplii, re-suspended in a new sterile 1 L glass bottles that contained sterile artificial sea water, and then again exposed to the same concentration of *V. campbellii* for another 3 days. On 14, 15 and 16 dph, the nauplii were washed in a similar way as described above to remove the bacteria. The control group, unexposed to *V. campbellii*, went through the same handling process. The *V. campbellii* exposure to *Artemia* nauplii was continued for 13 days ahead of the reproductive period (under standard laboratory conditions, *Artemia's* uterus develop on day 16 posthatching). Only early life stages of *Artemia* were exposed to *V. campbellii* to ensure that the uterus carrying the cysts/embryos was not directly exposed to the pathogenic stress conditions. On 17 dph, the salinity of the rearing water was increased from 35 g/L to 60 g/L in order to inhibit the

growth of *V. campbellii* and after 2 days, the salinity was further increased to 80 g/L to instigate cysts production.

Approximately 28 days posthatching, adult females (F0 generation) in both control and treatment started producing cysts (F1 generation). The F1 cysts from 3 replicates were isolated, pooled, and after terminating diapause by proper conditioning, were axenically hatched as described above. A major part of the emerged F1 nauplii were further cultured to maturity, after which the F2 nauplii were collected. The experiment was continued until, and including, the F3 generation. During the culture period, live algae were fed *ad libitum* every day, and to minimize bacterial exposure/contamination, sterile glass bottles and sterile artificial seawater were used. The detailed experimental design is shown schematically in **Fig. 5.1.**

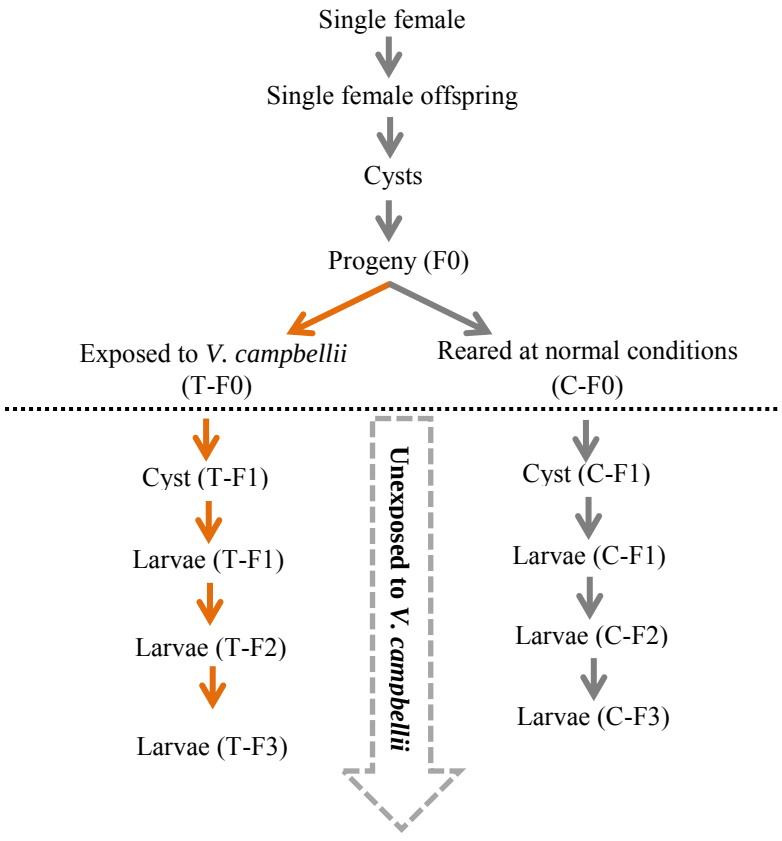

Fig. 5.1: Scheme of the experiment. A single female parthenogenetic *Artemia* was propagated to the next generation (i.e., single female offspring) under normal growth conditions. The single female offspring (all females) were reared normally until adulthood, and were then induced to produce cysts (F0). On hatching, the F0 progeny were divided into 2 groups. One group was exposed to *V. campbellii* ahead of the reproductive period (T-F0) as described in the methodology. The other group was grown

unexposed, under normal culture conditions 28 °C (C-F0). Approximately 28 days posthatching, the parental (F0) females from the treatment (T-F0) and control (C-F0) groups produced their next generation cysts i.e., T-F1 and C-F1, respectively. The cysts were hatched to produce their corresponding larvae i.e., T-F1 and C-F1. The F1 larvae from both groups were further cultured isothermally at 28 °C, without being given *V. campbellii* exposure, to maturity, after which the F2 larvae were collected. The experiment was continued until, and including, the F3 generation.

An aliquot of the emerged (F1 to F3) nauplii at instar II stage collected during each generation were subjected to *V. campbellii* challenge tests as described previously (**Chapter 3**). In brief, groups of 20 larvae were transferred in 7 replicates into separate sterile 40-mL glass tubes that contained 30 mL of 35 g/L sterile seawater. The nauplii were challenged with *V. campbellii* at 10^7 cells/mL. The survival of *Artemia* was scored at every indicated time intervals by counting the live *Artemia* as previously described (**Chapter 3**).

At each generation, survival data were analysed for statistical differences by subjecting the data to logistic regression analysis using GenStat (VSN international) version 16. Significance level was set at $P < 0.05$.

Results and discussion

In this study, we tested the phenomenon of multigenerational immune priming in an invertebrate, with the aim to seek evidence of adaptive-like immunity in invertebrates. For that, we exposed a population produced by an apomictic parthenogenetic *Artemia* i.e., lineages derived from a single mother, to a specific bacterial strain and evaluated the resistance of the offspring for three successive generations towards the same bacterial strain. Our results showed that the T-F1 offspring of F0 *Artemia* that were exposed at early stages to *V. campbellii* exhibited a significantly higher survival when challenged to *V. campbellii* than did the respective progeny of control C-F1 *Artemia* (**Fig. 5.2A**). Interestingly, this relatively higher survival extended to *Vibrio*-challenged T-F2 and T-F3 progenies, whose grandparents and great grandparents, respectively, received a *V. campbellii*-based immune-challenge during their early stages (**Fig. 5.2B & 5.2C**). In contrast to earlier studies showing that immune priming provides protection against

bacteria both within generation (Sadd and Schmid-Hempel, 2006; Pham et al., 2007; Roth et al., 2009) and transgenerationally till F1 generation (Little et al., 2003; Wu, et al., 2002; Moret, 2006), our study provided the first evidence indicating that immune priming effect confers protection to offspring against bacterial challenge across three subsequent, naive generations.

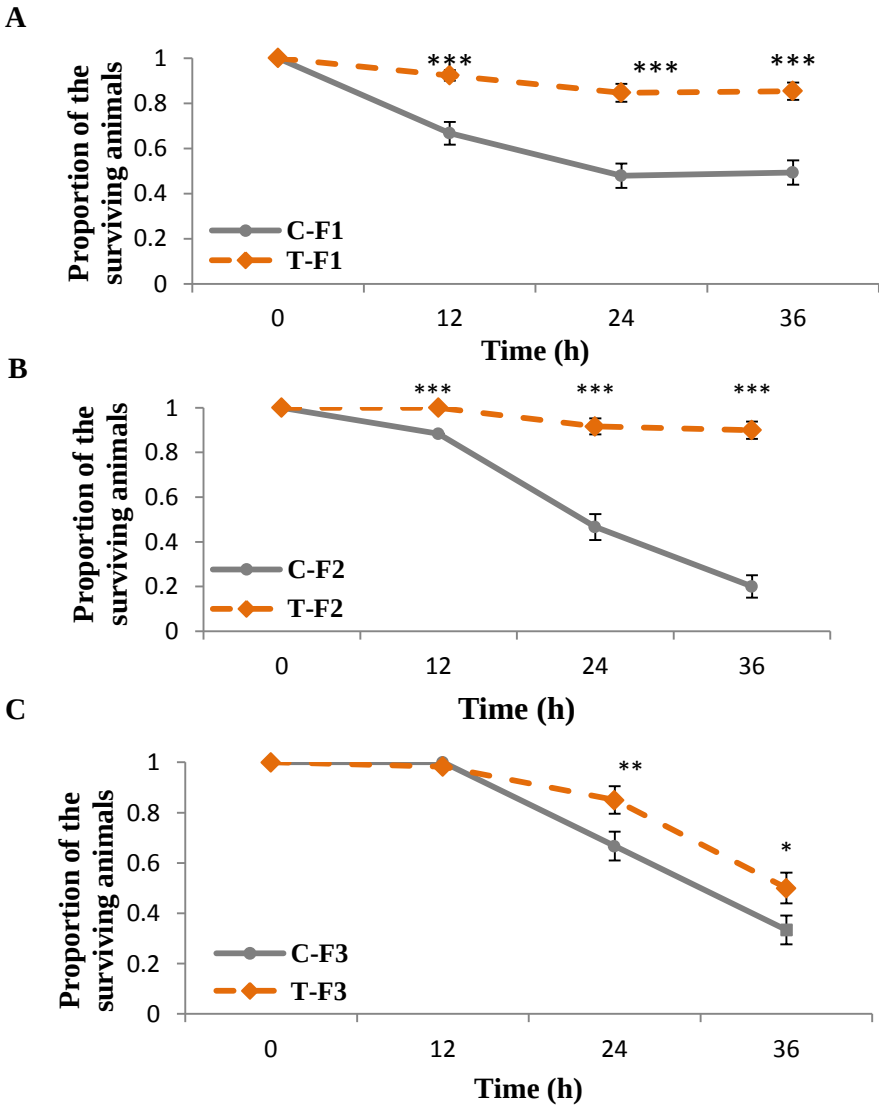

Fig. 5.2: Proportion of the survived *Artemia* larvae challenged with *V. campbellii*. For the treatment (T) and control (C) groups, refer to Fig. 1 for explanation. At every generation, the ovoviviparously produced larvae (at instar II stage) (A, B and C) were challenged with *V. campbellii* at 10^7 cells/mL and survival was scored at every 12 h intervals. Values are represented as means ± SE (n = 7). Asterisks represent a significant difference between the control and treatment of the respective generation * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

Interestingly, this phenomenon was observed in the parthenogenetic *Artemia*, which are having apomictic breeding behavior. Apomixis implies that a female parthenogenetic *Artemia* population clone has no other mechanism for genotypic change but mutation, which may induce genetic differentiation (Abatzopoulos, 2003). It is therefore unlikely that this observed multigenerational immune priming effect in *Artemia* results from selection on the parental population. However, at this point, we also cannot exclude the possibility of a genetic mutational event behind the observed phenotypic responses (Mager, 2006).

In the invertebrates, the underlying mechanisms that confer multigenerational resistance through immune priming are unclear yet. In the vertebrates, having the well-developed adaptive immune system, the immune-challenged mother confers immunity to immune naïve offspring by transferring immune factors (Bernardo, 1996). It is therefore plausible that invertebrate mothers exposed to pathogens imbue their eggs with their immune system peptides, and these peptides are transmitted to subsequent generation progenies, inducing resistance in the offspring against subsequent bacterial challenge. In fact, in the invertebrate bumblebees elevated antibacterial activity in offspring following priming of mother queens was shown to be associated with immune factors transmitted to the offspring via the eggs (Sadd and Schmid-Hempel, 2007). One can also argue that some bacteria or bacterial fragments from the challenged parents might have passed to the offspring through the eggs and stimulate the progeny's immunity. However, this is pure speculation and needs further investigation.

In recent years, a few studies have indicated that epigenetic regulatory mechanisms (such as DNA methylation/acetylation, histone modifications) are central elements in immune system function, allowing an appropriate gene expression pattern in immune cells in response to internal or external environmental cues, including pathogenic factors (see review Fernández-Morera et al., 2010). In a previous study, we have demonstrated that, on exposure to environmental heat stress, a parental population of parthenogenetic *Artemia* experiences an increased resistance against pathogenic *V.* campbellii and this acquired phenotypic traits were transmitted to three successive generations, none of them

were exposed to the parental stressor (**Chapter 3**). Results also suggested that the transgenerational inheritance of the increased resistance traits was associated with alteration in the levels of epigenetic marks, such as global DNA methylation and histones H3 and H4 acetylation level (**Chapter 3**). It is worth mentioning that the same phenotype was displayed by T-F1 to T-F3 offspring in response to immune challenge of the T-F0 parents in the present study (**Fig. 5.2**). It is therefore possible that the effect seen across generations in the present study may be a result of (transgenerational) epigenetic effects on the offspring's innate immune responses. Evidence for (transgenerational) epigenetic effects on innate immunity is mostly lacking in invertebrates. However, to our knowledge, there has been one study in bumblebee, where it was speculated that increased offspring resistance, when their mother queen received an immune challenge, is due to epigenetic effects on immunity (Sadd et al., 2005). Unfortunately, in that study no measurements of epigenetic marks or of immune parameters were made in the animals. These preliminary findings/speculation need to be substantiated by conducting mechanistic studies on the epigenetics of immune system/traits in invertebrates. Such studies will clarify our understanding of the nature of the invertebrate immune system and the role of inheritance.

Although mechanistically very different to vertebrate adaptive immunity, our results suggest that the immune system of invertebrate *Artemia* has the capacity to adapt in response to previous encounters with bacteria as manifested by increased offspring resistance across successive generation. The transmissions of immune factors from the immune-challenged mother to the offspring, via the eggs, can plausibly explain the observed multi-generational immune priming effect. Currently, we are investigating whether epigenetics mechanisms, such as DNA methylation and histone acetylation also play a role in multi-generational immune priming.

CHAPTER 6

Probing the Immunological and Molecular Mechanisms of Transgenerational Immune Priming Phenomenon by Using Invertebrate *Artemia franciscana* and *Vibrio campbellii* as Host-Pathogen Model

Abstract

The brine shrimp *Artemia*, like other invertebrates, is believed to rely only on its innate immune system, which does not exhibit specificity and memory, to fight against invading pathogens. However, some studies reported occurrence of adaptive-like immunity in insects that is termed immune priming and is broadly defined as increased protection to a pathogen following previous exposure to the same pathogen or an immune elicitor. This immune priming phenomenon has been suggested to be transgenerational i.e. primed parents bestow their next generation progenies with increased resistance against subsequent challenge with same or different pathogen. In spite of these phenomenological evidences, the immunological and molecular mechanisms behind such transgenerational immune priming in invertebrates remained elusive. In this study, we provided evidences suggesting that priming parental *Artemia* by exposing to pathogenic *Vibrio campbellii* protects their progenies against the same bacteria for three subsequent generations, but not against another strain of Vibrio (H6). Interestingly this increased resistance in three subsequent generations progenies against *V. campbellii* was positively correlated with elevated levels of the signaling molecules *hsp70* and *hmgb1* mRNA, suggesting the possible role of these molecules in mediating enduring resistance to *V. campbellii* challenge. No linear relationship between the immunity-related genes, except for peroxinectin immune gene, and the observed acquired resistance traits in the three successive generations progenies, whose ancestors were immune challenged were observed. Furthermore, results also indicated that the levels of histone H4 acetylation and H3K4Me3 are altered transgenerationally in the group whose ancestors were primed.

However, no mechanistic links could be established among the observed epigenetic modifications, immune-related genes and acquired resistance phenotypes.

Introduction

Adaptive immunity is an exclusive hallmark of the vertebrate immune system which provides the immune cells with the ability to recognize and proliferate in the presence of specific antigens. This immune system is characterized for having specificity and memory which is described as the ability to respond rapidly upon re-exposure to a particular antigen (Rowley and Powel, 2007). The invertebrates on the other hand are thought to lack adaptive immunity and specificity in their interactions with pathogens as they lack the system of clonal expansion of antigen-specific lymphocytes - machineries that mediate vertebrate adaptive immune responses (Hoffmann et al., 1999; Aderem and Ulevitch, 2000; Janeway and Medzhitov, 2002). Alternatively, with only innate immunity, invertebrate hosts are believed to act naively to each new encounter with pathogens (Hoffmann et al., 1999; Aderem and Ulevitch, 2000; Arala-Chaves and Sequeria, 2000; Mota et al., 2001; Janeway and Medzhitov, 2002). However, recent studies have indicated that invertebrates defense system have immunological specificity and memory of a kind that is a functional equivalent of adaptive immunity in vertebrates (reviewed by Little et al., 2005; Kurtz, 2005). This phenomenon of specificity and adaptivity in invertebrate was first shown by Kurtz and Franz (2003) in copepods, where a significant reduction in the reinfection rate was observed in copepods that had previously been exposed to the same subgroup of parasites. Highly specific invertebrate immune responses against specific pathogens were subsequently reported in other invertebrates, such as bumblebee (*Bombus terrestris*), fruit fly (*Drosophila melanogaster*) mosquito (*Anopheles gambiae*) and meal moth (*Plodia interpunctella*) (Watson et al., 2005; Sadd and Schmid-Hempel, 2006; Dong et al., 2006; Tidbury et al., 2011). Similarly, in shrimp, a prior challenge with a homologous viral protein provides better protection against the viral infection than previous exposure to a different heterologous viral protein (Witteveldt et al., 2006). In view of all this new evidence, it can be considered that the (innate) immune system of invertebrates may possess a certain degree of specificity and memory similar to a kind of vertebrate adaptive immunity (Johnson et al., 2008). However, to set it apart from the 'memory' known in vertebrates, the

'memory'-like phenomenon in invertebrates are termed as 'immune priming' and is broadly defined as increased or acquired protection against a pathogen following previous exposure to a pathogen or an immune elicitor (Schmid-Hempel, 2005). A few studies have indicated that the immune priming effect in invertebrates can extend for days and weeks, sometimes for almost the lifetime of the adult (Moret and Siva-Jothy, 2003; Korner and Schmid-Hempel, 2004). Other studies have indicated that similar to vertebrate adaptive immunity, the immune priming phenomenon in invertebrates can be transgenerational i.e., the protective/immune responses are transmitted from parents to offspring (Little et al., 2003; Sadd et al., 2005; Tidbury et al., 2011; Freitak et al., 2014). Despite these (phenomenological) observations, a comprehensive understanding of the 'phenomena of immune priming' in invertebrates still remains unclear. It is because most of the earlier studies addressing immune priming in invertebrates failed to make direct assessment of the immunological parameters and linked it to the functional responses (i.e., resistance towards homologous or heterologous pathogens) of the hosts, and/or did not support their observations by detailed explanation of the underlying biochemical and molecular mechanism(s) involved. Additionally, in case of studies on transgenerational immune priming in invertebrates, assessment of the maternally/paternally-transferred acquired immune responses or disease-resistant traits were carried out only in the first-generation progeny. If there is immune memory/specificity in invertebrates, it may be expected that the acquired memory-like immune responses or disease-resistant traits in the parental generation are transmitted across successive generations. However, at present, there is no evidence in the literature that unequivocally demonstrates the phenomenon of (multigenerational) immune priming in invertebrates, with a description of the likely mechanism(s) underpinning the phenomenon.

There is evidence indicating that epigenetic regulatory mechanisms are a central element in immune system function, allowing an appropriate gene expression pattern in immune cells in response to internal or external environmental cues, including pathogenic factors (see review Fernández-Morera et al., 2010). Some of these mechanisms have been elucidated and include DNA methylation (Verhoeven et al., 2010), histone modifications

and chromatin-remodeling proteins (Bannister and Kouzarides, 2011), and DNA silencing by non-coding RNAs (Storz, 2002). Epigenetic modifications have been shown to underlie information storage during innate immune memory in both plants (Jaskiewicz et al., 2011) and mammals (Chen et al., 2014; Saeed et al., 2014), however, whether epigenetic regulation of the immunological pathways are the mechanistic basis for immune priming phenomenon in invertebrates remained unknown.

Here, using the well-developed host-pathogen laboratory model system, the brine shrimp *Artemia franciscana* (an aquatic invertebrate) and its pathogenic bacteria *Vibrio campbellii* strain LMG21363 (**Chapters 3 & 4**), we first aimed to investigate the phenomenon of immune priming in invertebrate by carrying out a multigenerational study, and then subsequently verified whether the underlying mechanism of immune priming was represented by epigenetic programming through histone modifications.

Materials and methods

Experimental animal

Brine shrimp *Artemia franciscana* cysts (dormant embryos) originating from the Great Salt Lake, Utah, USA (EG® Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were used to produce the parental generation. To avoid any possible microbial contamination from the previous environment, *Artemia* cysts were hatched under axenic (germ-free) conditions following decapsulation and hatching procedures as described previously (Baruah et al., 2009). The sterile decapsulated embryos were transferred to 2 L glass bottles containing 35 g/L of sterile artificial seawater. Following incubation at 28 °C under constant illumination for 48 h (or day 2 post-hatching), the emerged instar II larvae (developmental stage at which mouth is open for ingestion of food) were used for experimental treatments.

Challenge of parental (F0) generation

A group of instar II larvae (day 2 post-hatching) was distributed in two groups (treatment and control), each with 3 replicates. Each group containing 4000 animals was

maintained in a 2 L glass bottle containing sterile artificial seawater (35 g/L), maintained at 28 °C under constant illumination (approximately 27 μmol/m^2.s) and aeration. The treatment group (on day 2 post-hatching) was exposed to 10^7 cells/mL concentration of pathogenic *V. campbellii* strain LMG21363 for a period of 4 days. On day 6 post-hatching, the live larvae from the bottles were collected over a sieve (250 μm), rinsed several times with sterile seawater to wash away the bacteria associated with the larvae, re-suspended in a new sterile 2 L glass bottles that contained sterile artificial sea water, and then allowed to recover for 3 days. On day 10 post-hatching, the larvae were washed in a similar way as described above and then exposed to the same concentration of *V. campbellii* for another 4 days. The control group, unexposed to *V. campbellii*, went through the same handling process. The *V. campbellii* exposure to *Artemia* larvae was continued till day 14 post-hatching ahead of the reproductive period (under standard laboratory conditions, *Artemia's* uterus start to develop on day 16 post-hatching). Only early life stages of *Artemia* were exposed to *V. campbellii* to ensure that the uterus carrying the cysts/embryos was not directly exposed to the pathogenic stress conditions. On day 17 post-hatching, the salinity of the rearing water was increased from 35 g/L to 70 g/L in order to inhibit the growth of *V. campbellii*. Under non-optimal environmental conditions, such as high salinity or low oxygen, *Artemia* switch from an ovoviviparous to an oviparous mode of reproduction (**Chapters 3 & 4**). On day 20 post-hatching, therefore, the salinity was further increased to 80 g/L to instigate cysts production by oviparous reproduction (**Chapters 3 & 4**). Throughout the culture period, the animals were fed *ad libitum* everyday with live green microalgae *Tetraselmis suecica* obtained from the Culture Collection of Algae and Protozoa (CCAP) Department (Dunstaffnage Marine Laboratory, Oban, UK).

Production and collection of the progenies as larvae and cysts

Approximately 26 days post-hatching, adult females (F0 generation) in both control and treatment produced larvae and cysts (F1 generation). As the F1 larvae were produced in the parental generation environmental conditions, they were discarded, and only the F1

cysts were used to produce the subsequent generation progenies. The deposited F1 cysts however remain in a state of diapause and are not ready for hatching (Drinkwater and Clegg, 1991; Nambu et al., 2008). They were therefore conditioned as described previously to terminate diapause (**Chapter 3**). A part of the activated F1 cysts was stored at 4 °C for further use, while the remaining part was hatched axenically as described above to avoid bacterial contamination from parental generation. The emerged F1 larvae were further grown to maturity, after which the F2 cysts and larvae were collected. The experiment was continued until, and including, the F3 generation. During the culture period, live algae were fed *ad libitum* every day, and to minimize bacterial exposure/contamination, sterile glass bottles and sterile artificial seawater were used. The detailed experimental design is shown schematically in **Fig. 6.1**.

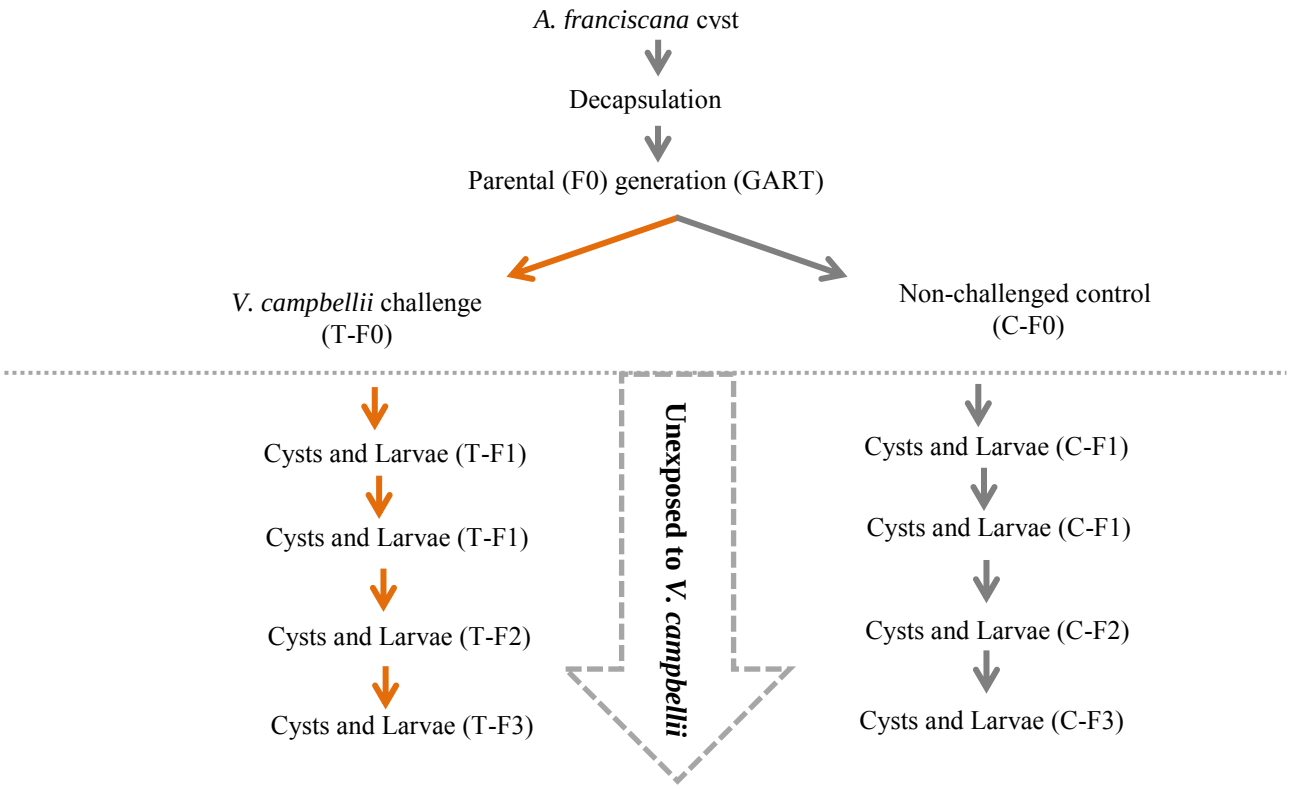

Fig. 6.1: Scheme of the experiment. On hatching the *A. franciscana* cysts under axenic condition, the parental F0 generation larvae were obtained. Animals from parental generation were divided into 2 groups. One group was exposed to *V. campbellii* ahead of the reproductive period (T-F0) as described in the methodology. The other group was grown unexposed, under normal culture conditions 28 °C (C-F0). Approximately 28 days post-hatching, the parental (F0) females from the treatment (T-F0) and control (C-F0) groups produced their next generation cysts i.e., T-F1 and C-F1, respectively. The cysts were hatched to produce their corresponding larvae i.e., T-F1 and C-F1. The F1 larvae

from both groups were further cultured isothermally at 28 °C, without being given *V. campbellii* exposure, to maturity, after which the F2 larvae were collected. The experiment was continued until, and including, the F3 generation.

An aliquot of the larvae collected during each generation was subjected to bacterial challenge tests as described below. This entire procedure of pathogen challenge at F0 generation followed by offspring production and challenge assays was repeated once to confirm the reproducibility of the results. The detailed experimental design is shown schematically in **Fig. 6.1**

Bacterial strains and growth conditions

Two pathogenic bacterial strains *Vibrio campbellii* strain LMG21363 and *Vibrio* strain H6 were used in the *Artemia* challenge study. Both these strains are Gram-negative bacteria that are ubiquitous in the marine environment. The *V. campbellii* strain LMG21363, obtained from the stock cultures of the Lab of Aquaculture & *Artemia* Reference Center, Ghent University, were known to cause life threatening vibriosis (bacterial disease) in the wild and cultured aquatic animals including in *Artemia*. The strain H6 was obtained from a hatchery in Rio Grande do Norte and was originally isolated from pacific white shrimp *Penaeus vannamei* (Natal-Area, Brazil). These two strains were selected because both *V. campbellii* and H6 are from the same clade (Vanmaele et al., 2014) and therefore, the specific immune response can be separated from the general innate immune response of the animals. Both the strains, stored in 40% glycerol at -80 °C, were incubated at 28 °C for 24 h on Marine Agar 2216 (Difco Laboratories, Detroit, MI, USA) and then grown to log phase in Marine Broth 2216 (Difco Laboratories, Detroit, MI, USA) by incubation at 28 °C with shaking. The bacteria were transferred to a sterile tube, centrifuged at 2200 x *g* for 15 min at 28 °C, suspended in filtered (0.2 µm) sterile seawater, and immediately used in challenge experiments. Bacteria cell numbers were determined spectrophotometrically at 550 nm according to McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an optical density of 1.000 corresponds to 1.2×10^9 cells/mL (Baruah et al., 2010).

Survival assay of Artemia progenies

An aliquot of the emerged (F1 to F3) larvae collected at every generation was subjected to bacterial challenge tests as described previously (**Chapter 5**). In brief, groups of 30 larvae were transferred in 7 replicates into separate sterile 40-mL glass tubes that contained 30 mL of 35 g/L sterile seawater. The larvae were challenged with *V. campbellii* or H6 strain at 10^7 cells/mL. The survival of *Artemia* was scored at every indicated time intervals.

The *Artemia* cysts from the control and treatment groups collected from all the three generations were hatched simultaneously under axenic conditions as described previously (Drinkwater and Clegg, 1991; Baruah et al., 2012) to run a common garden challenge experiment. A required number of the emerged age- and size- synchronized larvae were challenged with *V. campbellii* or H6 strain as described above. The remaining numbers were further reared under standard laboratory conditions (in triplicates) till juvenile stage (18 days old). Once at juvenile stage, animals were sampled in 3 replicates, rinsed in sterile distilled water, immediately frozen in liquid nitrogen, and stored at -80 °C for analysis of immune-related genes and epigenetic marks.

Cyst and larvae production phenotypes

The animals from F1 to F3 generation of both control and treatment groups grown under common garden conditions were tested for their reproductive phenotypes i.e. production of cysts and larvae. To this end, eight mating couples from every generation of both control and treatment groups were transferred to Falcon tubes that contained 80 g/L seawater. The production of cysts and larvae from each couple were monitored twice a week for a period of 3 weeks after their first reproduction.

RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNA was extracted from the *Artemia* samples using the SV total RNA isolation kit (Promega, Belgium). First strand cDNA was synthesized from 1 µg total RNA using the RevertAid™ H minus First strand cDNA synthesis kit (Fermentas Gmbh, Germany) following the manufacturer's guidelines. The expressions of heat shock protein 70

(*hsp70*), high mobility group box 1 protein (*hmgb1*), *beta-1,3-glucan-binding protein* (*lgbp*), down syndrome cell adhesion molecule (*dscam*), prophenoloxidase (*propo*), transglutaminase (*tgase*), *ferritin (ftn)* and peroxinectin (*pxn*) genes in the larvae were analyzed by qPCR using a pair of specific primers. Some of these innate immune genes were recently identified in *Artemia franciscana* based on their homology with crayfish (Vanmaele, 2015). The qPCR amplifications were carried out in a total volume of 25 µL, containing 9.8 µL of nuclease free water, 0.4 µL of each primer, 12.5 µL of Maxima SYBR Green qPCR Master mix (Fermentas Gmbh, Germany) and 2 µL of cDNA template. The qPCR was performed in a One Step qPCR instrument (Applied Biosystems) using a four-step amplification protocol: initial denaturation (10 min at 95 °C); 40 cycles of amplification and quantification (15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C); melting curve (55-95 °C with a heating rate of 0.10 °C/s and a continuous fluorescence measurement) and cooling (4 °C). The *β-actin* gene was used as a reference gene for standardizing the expression of target genes. Master mixes were prepared in duplicate for each biological replicate of the sample and qPCR for target and reference genes was performed. Relative quantification of target gene transcripts with a chosen reference gene transcript was done following the Pfaffl method with the Relative Expression Software tool (REST©) (Pfaffl et al., 2002; Baruah et al., 2011).

Total histone extraction

Histone extraction of *Artemia* samples was carried out using the EpiSeeker histone extraction kit (ab113476; Abcam, Cambridge, UK) according to the manufacture's instruction. Histone concentration was determined following the Bradford method using bovine serum albumin as standard (Bradford, 1976).

Western blot analysis of histone H3K4me3

Histone samples were combined with loading buffer, vortexed, heated at 95 °C for 5 min, and then electrophoresed in 10% SDS-PAGE gel, with each lane receiving equivalent amounts of protein (15 µg). HeLa (heat-shocked) cells (6 µg; Enzo Life Sciences, USA)

were loaded in one well to serve as a positive control and for further quantification of the H3K4me3 in the samples. Gels were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Belgium) for antibody probing. Membranes were incubated with blocking buffer (50 mL of 1X phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin) for 60 min at room temperature and then with rabbit polyclonal anti-histone H3 (tri methyl K4) antibody (ab8580; Abcam, Cambridge, UK), which is sensitive to histone H3 trimethylation, at the recommended dilution of 1 µg/mL. Goat anti-rabbit secondary antibody (Sigma-Aldrich, Belgium) at the recommended dilution of 1:3000. The membranes were then treated with enhanced chemiluminescence reagent (GE healthcare, UK) and the signals were detected by a ChemiDoc MP Imaging System (Bio-Rad, Belgium). The relative signal intensity was quantified by densitometry with Bio-Rad Image Lab™ Software version 4.1.

Analysis of histone H3 and H4 total acetylation

Histone (3 µg) samples from all the generations (F0 to F3) of the control and treatment groups were analyzed in three biological replicates for histone H3 and H4 total acetylation using the EpiSeeker histone H3 (ab131561; Abcam, Cambridge, UK) and histone H4 total acetylation detection fluorometric kits, respectively (ab131562; Abcam, Cambridge, UK) according to the manufacturer's instructions.

Statistical analysis

Survival data were subjected to logistic regression analysis using GeneStat 16 (VSN International, Hemel Hempstead, UK) to determine significant differences between the control and treatment. The mRNA expression was calculated using Pfaffl method (Pfaffl, 2002). Results for target gene mRNA quantification are presented as fold expression relative to *Artemia* actin. The expression level in control was regarded as 1.0 and thereby the expression ratio of the treatments was expressed in relation to the control. To analyze significant differences between the control and treatment the obtained data was subjected to Student's *t*-test using Statistical Package for the Social Sciences (SPSS) 19.0 (IBM,

Armonk, NY, USA). Significant differences in the epigenetic marks (histone H3 and H4 acetylation and H3K4Me3) between the control and the treatment at each generation were determined by similar Student's *t*-test. Significance level was set at $P < 0.05$.

Results

Progeny of V. campbellii-exposed ancestors exhibit increased resistance against challenge with the same bacterial strain in the 3 successive unexposed generations

To determine the phenomenon of adaptive-like immunity in the invertebrate *Artemia*, we first analyzed the phenotype of induced resistance in the F1 progeny, produced ovoviviparously, towards pathogenic *V. campbellii* following immune challenge of the parental F0 population with the same pathogenic bacterial strain. As shown in **Fig. 6.2A**, the unexposed T-F1 progeny derived from T-F0 *Artemia* exposed repeatedly at early stages to *V. campbellii* exhibited increased resistance towards *V. campbellii* challenge, as was evidenced by significantly higher survival than the respective progeny of control C-F1 *Artemia*. To examine the durability of the transgenerational resistance, the F1 progeny from both control and treatment groups were grown till adult under stress-free (no *Vibrio*-based immune challenge) conditions, and bred to produce F2 and F3 generations. The results showed that higher survival phenotype persisted even in the F2 and F3 progenies, whose grandparents and great grandparents, respectively, were exposed at early stages to *V. campbellii* (**Fig. 6.2B & 6.2C**). In the following experiment, we validated the results of the *Vibrio*-resistance test described above by carrying out a common garden experiment under gnotobiotic conditions. It is because, the axenic environment of test provides the opportunity to evaluate the phenotypes in absence of environmental microbial interference. In this experiment, *Artemia* cysts from all 3 (F1 to F3) generations were hatched simultaneously in an axenic way (Baruah et al., 2014), and the obtained axenic larvae were challenged with *V. campebellii* as described above. Such experiment and condition are useful because they can provide a more mechanistic understanding for the causes of phenotypic variation in the 3 successive generations in response to *V. campbellii*-based immune-challenge of the F0 generation. The results showed a similar

survival trend as observed for the animals from ovoviviparous reproduction (**Fig. 6.2D & 6.2F**). These results suggested that that *Artemia* immune response can adapt and provide long lasting protection against *V. campbellii* infection.

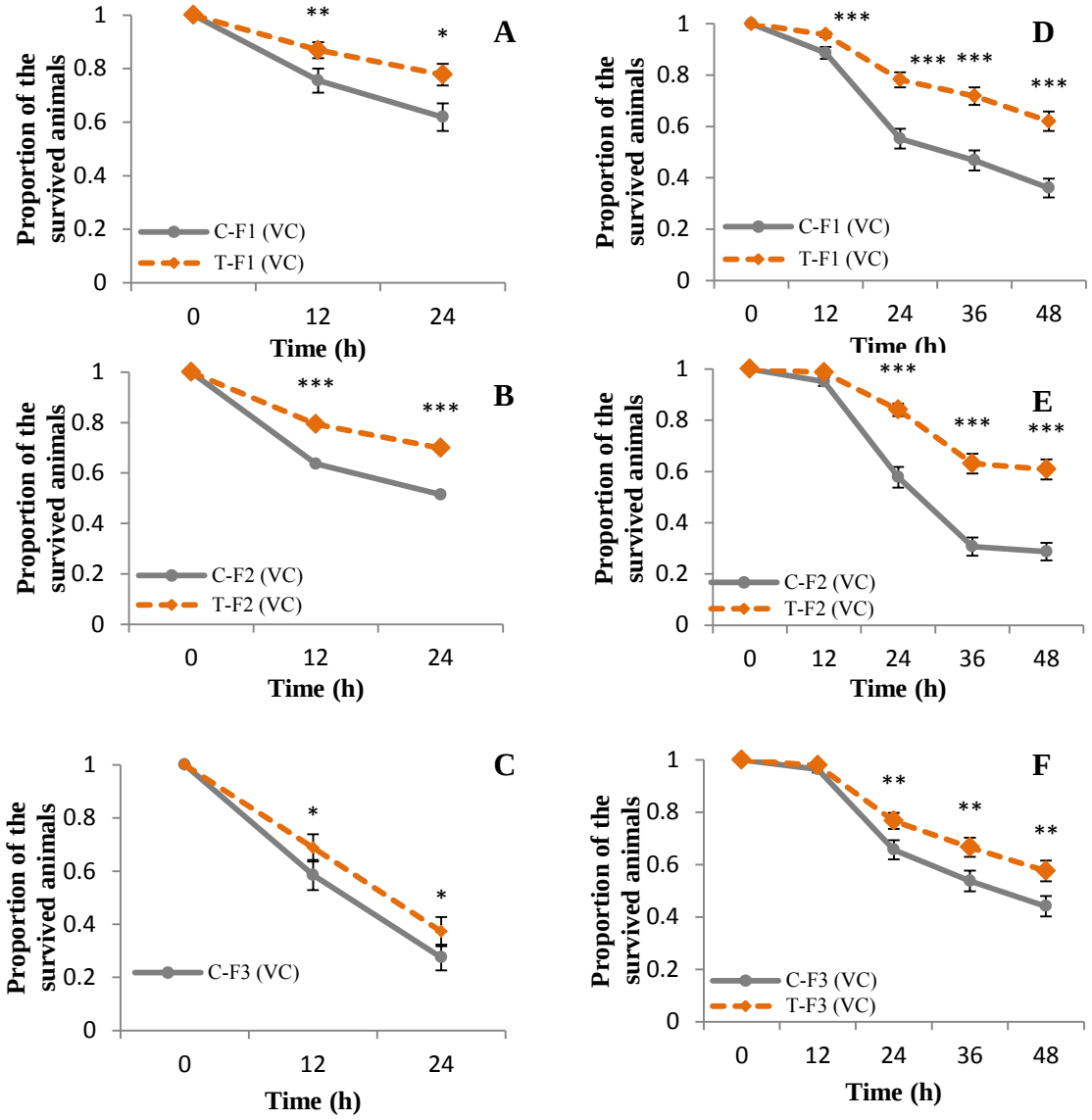

Fig. 6.2: Proportion of the survived *Artemia* larvae challenged with *V. campbellii*. For the treatment (T) and control (C) groups, refer to **Fig. 6.1** for explanation. The ovoviviparously (A, B and C) or common garden oviparously (D, E and F) produced larvae (at instar II stage) were challenged with *V. campbellii* at 10^7 cells/mL and survival was scored at every 12 h intervals. Values are represented as means ± SE (n = 7). Asterisks represent a significant difference between the control and treatment of the respective generation *($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

Progeny of V. campbellii-exposed ancestors exhibit no increased resistance against challenge with a different bacterial strain H6 in the 3 successive unexposed generations

Adaptive immunity is characterized by immunological memory and specificity (Kurtz and Franz, 2003). Next, we aimed to determine the specific memory of the defense system in *Artemia*. To this end, we carried out another challenge test to analyze whether the progeny from the F1 to F3 generations of *Artemia* (produced ovoviviparously) that were exposed to *V. campbellii* can induce resistance towards another pathogenic *Vibrio* strain H6. If there is a specific memory inherent in the defense of *Artemia* host, we would expect no increase resistance in the T-F1 to T-F3 progenies against H6 strain. Indeed, we found that the unexposed T-F1 progeny derived from T-F0 *Artemia* exposed to *V. campbellii* did not exhibit any significant improvement in their survival upon challenge with H6 compared to the same-generation progeny of control parents (C-F1 progeny) (**Fig. 6.3A**). Similar result was observed at the F2 generation (**Fig. 6.3B**). However, at F3 generation, the survival of the T-F3 progeny whose great grandparents were exposed to *V. campbellii* was significantly higher than that of the C-F3 progeny of control parents **(Fig. 6.3C**). Next, we confirmed the results of the above challenge test by conducting the experiment under common garden and gnotobiotic conditions. We found that none of the progenies from the F1 to F3 generations of *Artemia* that were exposed to *V. campbellii* could induce resistance against another pathogenic H6 strain, as was evidenced by no significant difference in the survival compared to the respective progenies of the control parents (**Fig. 6.3D & 6.3F**). These data from both the common garden experiment and the generation-specific challenge tests, together with the data from the *Vibrio*-resistance test reported above (see **Fig. 6.2 & 6.3**) suggest that *Artemia* primed response is specific and can persist for generations.

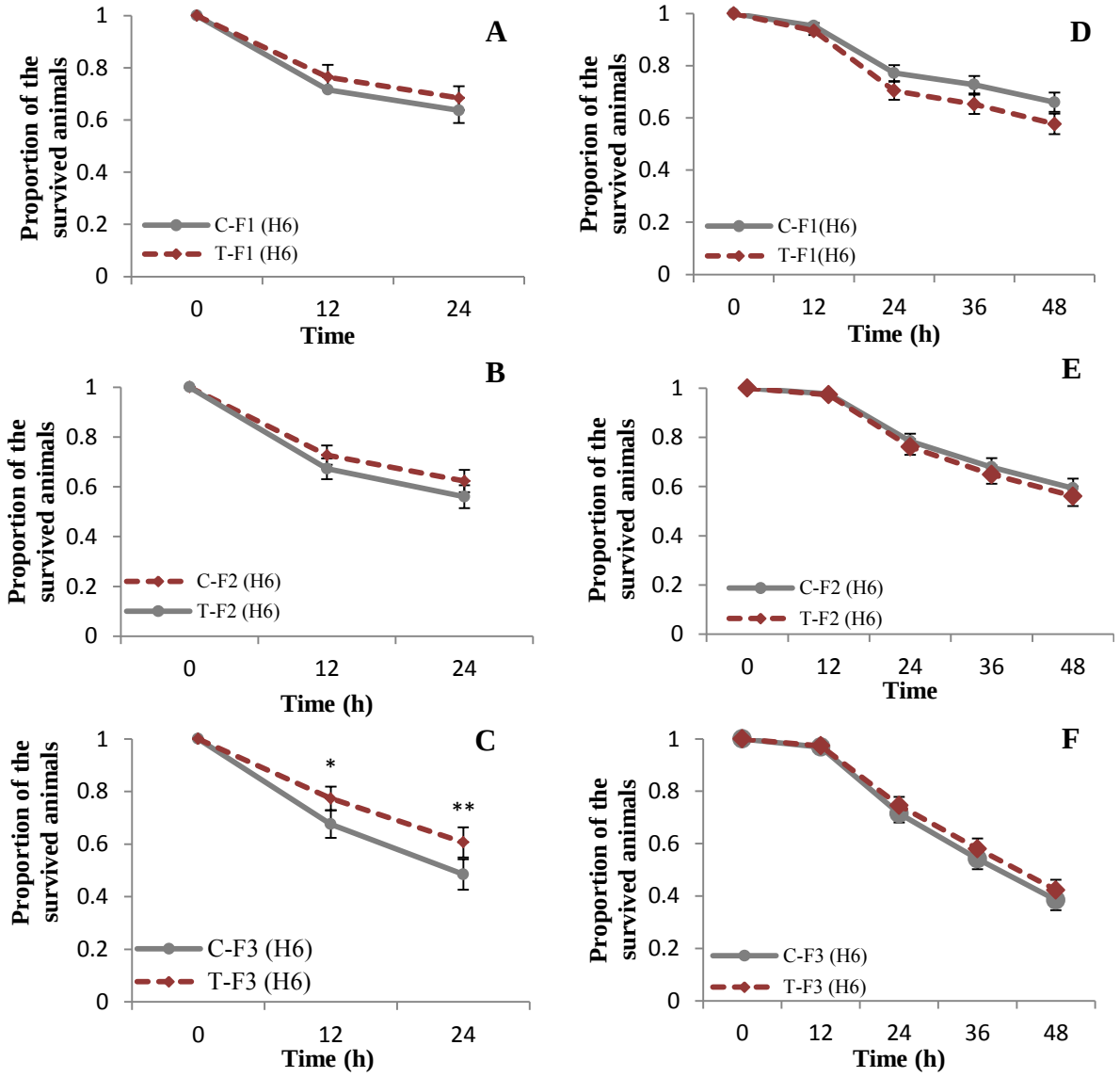

Fig. 6.3: Proportion of the survived *Artemia* larvae challenged with *H6*. For the treatment (T) and control (C) groups, refer to Fig. 6.1 for explanation. The ovoviviparously (A, B and C) or common garden oviparously (D, E and F) produced larvae (at instar II stage) were challenged with *V. campbellii* at 10^7 cells/mL and survival was scored at every 12 h intervals. Values are represented as means ± SE (n = 7). Asterisks represent a significant difference between the control and treatment of the respective generation *($P < 0.05$), ** ($P < 0.01$).

Immune challenge of the parental generation of Artemia influences the reproductive behavior of the progeny in the 3 successive generations

Previous studies reported that immune priming phenomenon in invertebrates costs energy and can causes marked effects on numerous life-history traits (e.g. fecundity,

development period, and egg size) of the animal both at the later part of life and in its progeny (Freitak et al., 2003; Roth et al., 2010; Moreau et al., 2012). We next investigated whether induction of immune priming by *V. campbellii* incurs reproductive costs that would manipulate the three successive generation fecundity. To this end, we measured reproductive behavior (i.e. the larval production and fecundity) of the progeny from the F1 to F3 generations of *Artemia* that were immune challenged with *Vibrio*. The results showed that the T-F1 to T-F3 female progenies, whose ancestors were immune challenged with *Vibrio*, produced significantly higher number of cysts (dormant eggs) than their respective progeny from the control ancestors (**Fig. 6.4A**). For the number of larvae produced ovoviviparously by the progenies from F1 to F3 generation, we did not observe any significant difference between the control and treatment progenies at the F1 and F2 generation (**Fig. 6.4B**). However, interestingly this response appeared to reverse in the F3 generation, where the T-F3 progeny exhibited a significant 4-fold increase in the number of larval production compared to the C-F3 progeny of the control parental group (**Fig. 6.4B**). These results indicates that in response to *Vibrio* exposure, the parental *Artemia* adaptively adjust the reproductive phenotype of their progenies for 3 successive generations.

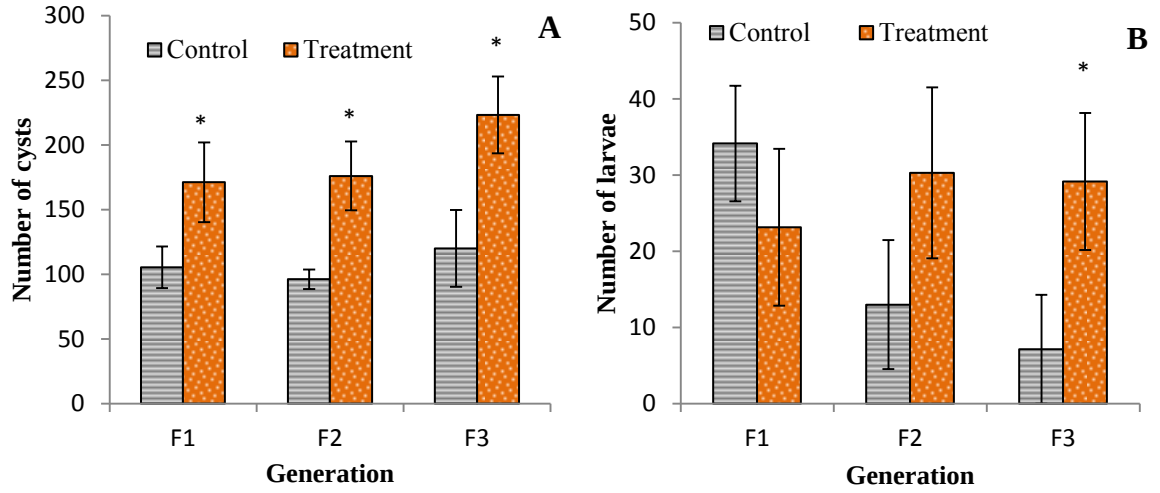

Fig. 6.4: Number of produced (A) Cysts and (B) by *Artemia* adults from three generations in common garden culture. For the treatment and control groups, refer to **Fig. 6.1** for explanation. Total cyst and larvae collected from F0 to F3 adults were counted for the period of 2 weeks. Details are provided in methodology. Values are represented as means ± SE (n = 8). Asterisks represent the significant difference between the control and treatment * ($P < 0.05$).

Progeny of V. campbellii-exposed ancestors has increased levels of the signal molecules heat shock protein 70 (HSP70) and high mobility group box 1 (HMGB1) in the 3 successive unexposed generations

Because the signaling protein molecules HMGB1 and HSP70 are associated with mounting protective immune responses against bacterial infection (**Chapter 4**; Baruah et al., 2014), we next sought to investigate whether these signal molecules are involved in the observed acquired memory-like resistance against pathogenic *V. campbellii*. We addressed this by analyzing the expression levels of *hsp70* and *hmgb1* genes by employing qPCR (details in Materials and Methods).

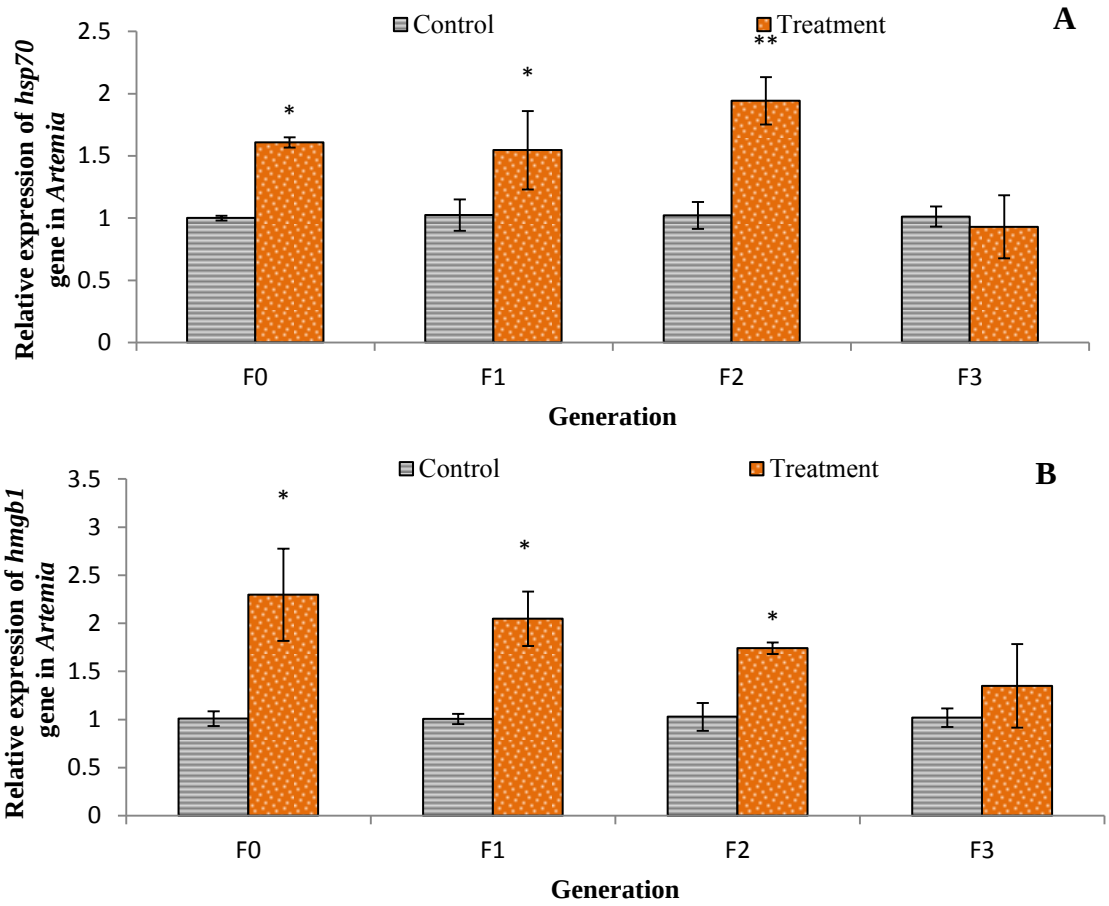

Fig. 6.5: Expression of signal molecules (A) *hsp70* and (B) *hmgb1* mRNA in the the parental *Artemia* and three successive generation progenies (common garden test). For the treatment and control groups, refer to **Fig. 6.1** for explanation. Expression of *hsp70* or *hmgb1* mRNA in the control group was regarded as 1.000. Results, which are the mean of 3 biological replicates, are presented relative to *Artemia* actin gene expression, according to the equation of Pfaffl et al. (2002). Bars indicate standard error from the mean. Significant differences between the treatment and control at different generation are indicated by * ($P < 0.05$).

As shown in **Fig. 6.5**, the mRNA transcript levels of *hsp70* and *hmgb1* in the *Vibrio*-exposed parental *Artemia* (T-F0) were significantly higher by respectively 1.6-fold ($P < 0.05$) and 2.3-fold ($P < 0.05$) relative to the unexposed C-F0 parental group. Increased levels of *hsp70* and *hmgb1* mRNA transcripts were also detected in the T-F1 (by 1.5- and 2-fold, respectively, $P < 0.05$) and T-F2 (by 1.9- and 1.7-fold, respectively, $P < 0.05$) progenies, whose ancestors were immune challenged with *Vibrio*. However, in the F3 generation, no significant difference was found in the transcript levels of *hsp70* and *hmgb1* mRNAs between the C-F3 control and T-F3 treatment groups. These results indicate that HSP70 and HMGB1 signaling molecules may be involved, at least to some extent, in induction of acquired memory-like resistance in the T-F1 to T-F3 progenies of immune challenged F0 animals.

Progeny of V. campbellii-exposed ancestors did not exhibit increased expression of down syndrome cell adhesion molecule (dscam) and lipopolysaccharide- and beta-1,3-glucan-binding protein (lgbp) genes in the 3 successive unexposed generations

Pattern recognition receptors (PRR) are involved in the first step of invertebrate immune response as they bind to highly conserved pathogen structures, such as peptidoglycans or LPS from bacteria, β-glucans from fungi, or to danger associated molecular patterns, such as HSPs and HMGB1 (Janeway and Medzhitov, 2002; Lotze and Tracey, 2005; Harris et al., 2012). Therefore, we investigated whether increased *hsp70* and *hmgb1* genes expression contribute to acquired *V. campbellii*-resistance traits in the T-F1 to T-F3 progenies of immune challenged F0 *Artemia* by inducing PRRs. Surprisingly, we observed no significant up-regulation of *dscam* gene, neither in the *Vibrio*-exposed T-F0 parental generation nor in their successive generation T-F1 to T-F3 progenies (**Fig. 6.6A**). The expression level of *lgbp* gene also did not upregulate significantly in any of the treatment (T-F0 to T-F3) groups compared to their respective controls (**Fig. 6.6B**). In contrast, the gene level in the T-F0 *Artemia* down regulated by 4.2-fold relative to the C-F0 group ($P < 0.05$). The T-F1 progeny of parents that received immune challenge also exhibited significantly lower expression level of this gene relative to the respective T-F1

progeny of the control parental group (1.5-fold, $P < 0.05$). In the T-F2 and T-F3 progenies, a trend toward a decrease in the expression level of *lgbp* gene relative to their respective controls was observed also, but the difference was not statistically significant ($P > 0.05$). These observations suggest that the immune receptors *dscam* and *lgbp* mRNA are not involved in mounting phenomenon of inherited acquired memory-like immune responses in the T-F1 to T-F3 progenies of immune challenged F0 *Artemia*.

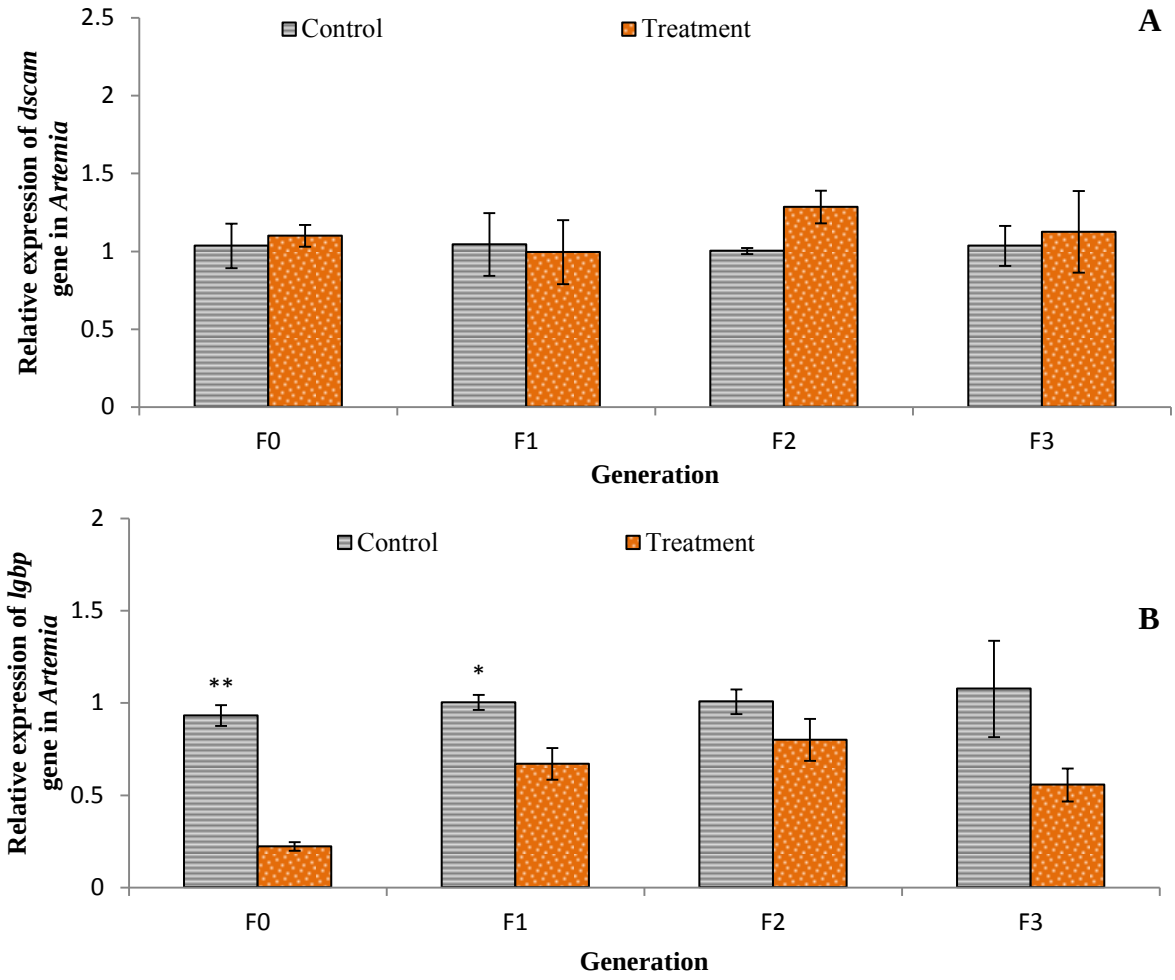

Fig. 6.6: Expression of receptor molecules (A) *dscam* and (B) *lgbp* mRNA in the the parental *Artemia* and three successive generation progenies (common garden test). For the treatment and control groups, refer to **Fig. 6.1** for explanation. Expression of *dscam* or *lgbp* mRNA in the control group was regarded as 1.000. Results, which are the mean of 3 biological replicates, are presented relative to *Artemia* actin gene expression, according to the equation of Pfaffl et al. (2002). Bars indicate standard error from the mean. Significant differences between the treatment and control at different generation are indicated by * ($P < 0.05$), ** ($P < 0.01$).

Progeny of V. campbellii-exposed ancestors exhibit substantial changes in the expression of innate immune-related genes in the 3 successive, unexposed generations

Next we investigated the contribution of humoral responses to the induction of acquired resistance traits in the T-F1 to T-F3 progenies of the F0 *Artemia*. To this end, we analyzed the expression of a set of four innate immunity-related genes i.e. prophenoloxidase (*proPO*), ferritin (*ftn*), transglutaminase (*tgase*) and peroxenectin (*pxn*) that were previously reported to be involved in inducing resistance in animals against bacterial infection (Park et al., 2010; Baruah et al., 2014). As shown in **Fig. 6.7A**, there was no significant upregulation of the *proPO* gene in the *Vibrio*-exposed parental T-F0 *Artemia* relative to the unexposed C-F0 parental group. In the F1 generation, progeny of the parents that received *V. campbellii*-based immune challenge exhibited a 2.3-fold increase in the expression level of *proPO* gene relative to the corresponding C-F1 control ($P < 0.01$). Surprisingly, the *proPO* expression level in the T-F2 progenies down regulated significantly ($P < 0.05$) by 1.5-fold relative to the control C-F2 progeny. In the F3 generation, the *proPO* transcripts in the T-F3 progeny exhibited no significant difference compared to that of the corresponding C-F3 control progeny.

Similar to what was observed for the *proPO* gene, the mRNA expression of *ftn* in the T-F0 parental *Artemia* remained at the same level as that in the C-F0 parental group (**Fig. 6.7B**). In the F1 generation, the T-F1 progeny exhibited a significant ($P < 0.05$) 1.8-fold increase in the *ftn* gene level relative to the control C-FI progeny. In the F2 generation, the expression level in the T-F2 progeny declined significantly by a factor of 1.4-fold. However, in the F3 generation, the expression level in the T-F3 progeny was the same as that in the respective C-F3 control.

The mRNA expression level of *tgase* in the T-F0 progeny remained unaltered in comparison to the respective C-F0 control progeny (**Fig. 6.7C**). However, in the F1 and F2 generations, the expression level in the T-F1 and T-F2 progenies increased significantly by respectively 1.8-fold ($P < 0.01$) and 1.2-fold ($P < 0.05$) relative to the respective C-F1 and C-F2 controls. In the F3 generation, no significant difference was

observed in the expression level of *tgase* mRNA between the C-F3 control and T-F3 treatment groups (**Fig. 6.7C**).

The mRNA expression levels of *pxn* in the *V. campbellii* exposed T-F0 parental *Artemia* was significantly higher (2-fold, $P < 0.05$) than in the unexposed C-F0 parental group (**Fig. 6.7D**). A significantly increased expression levels of *pxn* mRNA was also detected in the T-F1 to T-F3 progeny, whose ancestors were exposed to *V. campbellii*.

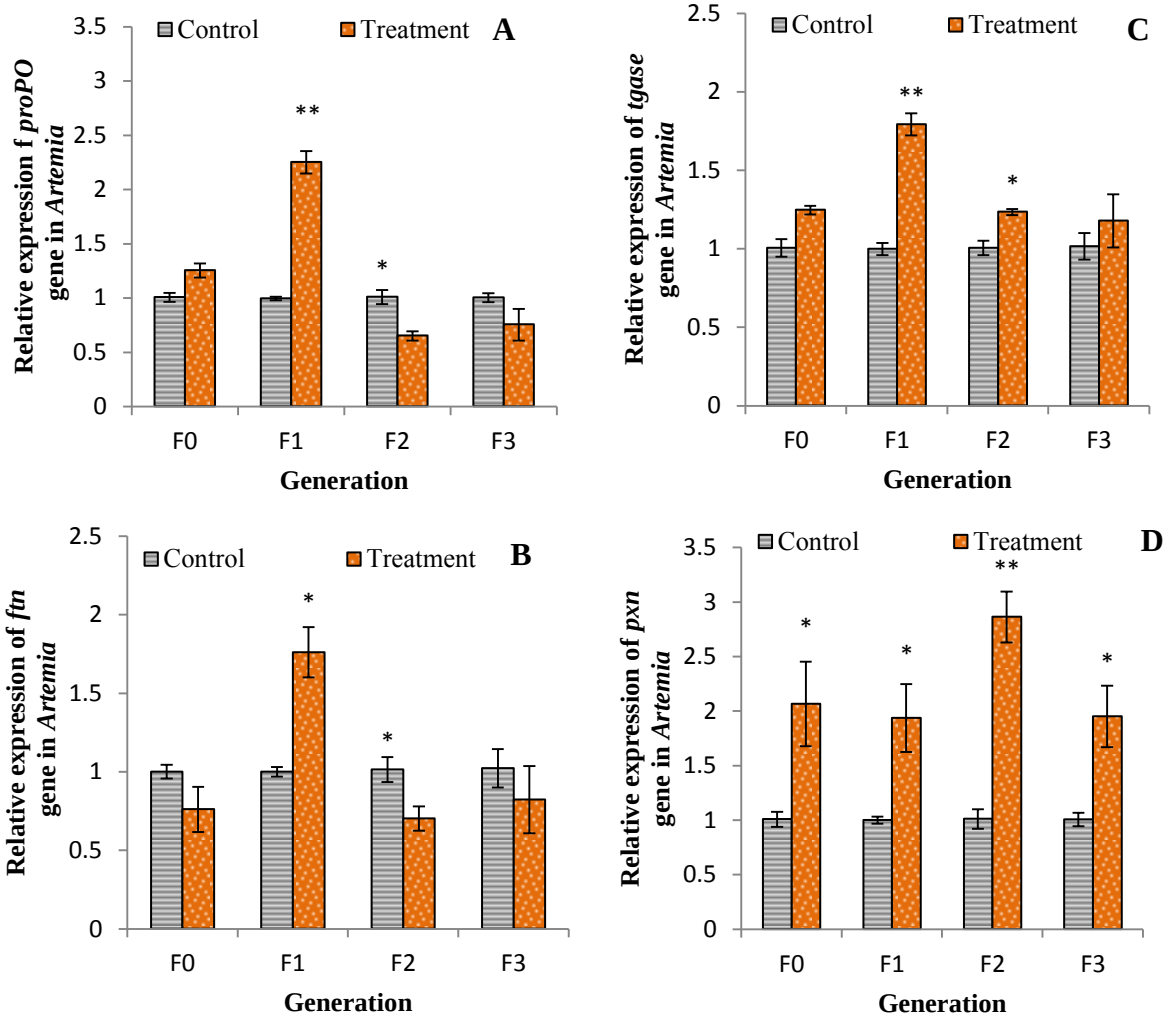

Fig. 7: Expression of immune molecules (A) *proPO,* (B) *ftn,* (C) *tgase* and (D) *pxn* mRNA in the parental *Artemia* and three successive generation progenies (common garden test). For the treatment and control groups, refer to Fig. 1 for explanation. Expression of *proPO, ftn, tgase* or *pxn*mRNA in the control group was regarded as 1.000. Results, which are the mean of 3 biological replicates, are presented relative to *Artemia* actin gene expression, according to the equation of Pfaffl et al. (2002). Bars indicate standard error from the mean. Significant differences between the treatment and control at different generation are indicated by * ($P < 0.05$), ** ($P < 0.01$).

The increase was by respectively 1.9-fold ($P < 0.05$), 2.7-fold ($P < 0.01$) and 2-fold ($P < 0.05$) at the F1, F2 and F3 generations relative to the respective controls. Taken together, these results suggest that in response to immune challenge of the parental *Artemia* with *V. campbellii* exposure, the *proPO, tgase* and *ftn* and *pxn* immune genes that are induced in a differentially manner both within the parental generational as well as in the 3 successive T-F0 to T-F3 generations may have contributed to acquired memory-like immune responses and consequently to *V. campbellii*-resistant traits.

Vibrio campbellii*-exposed *parental generation and the 3 successive unexposed generations exhibited stochastic pattern in the level of histones H3 and H4 acetylation

Previous studies have implicated the critical involvement of histone acetylation in the transcriptional (up)regulation of immune genes in animals (Chen et al., 2002; Thomson et al., 2004; Zhao et al., 2006; Fritah, 2009). We therefore investigated whether modification of histone H3 and H4 proteins is the underlying mechanism behind the observed transgenerationally inherited acquired protective immune responses. To address this possibility, we analyzed the acetylation level of histone H3 and H4 (details in Materials and Methods). As shown in **Fig 6.8A**, we observed no significant increase in the acetylation level of histone H3 protein, neither in the *Vibrio*-exposed T-F0 parental generation nor in the successive generation, unexposed T-F1 to T-F3 progenies. In regard to histone H4, a stochastic pattern of acetylation level was observed across T-F0 to T-F3 generations of *Artemia*. This means a relatively higher level of acetylated H4 in the T-F0 parental *Artemia* than in the C-F0 parental group (2.16-fold; $P > 0.05$), significantly lower level in the T-F1 progeny compared to the C-F1 control progeny (15-fold; $P < 0.05$), again considerably higher in the T-F2 progeny compared to C-F2 control progeny (1.9-fold; $P > 0.05$), and then significantly lower level in the T-F3 progeny compared to the corresponding control progeny (17-fold; $P < 0.05$).

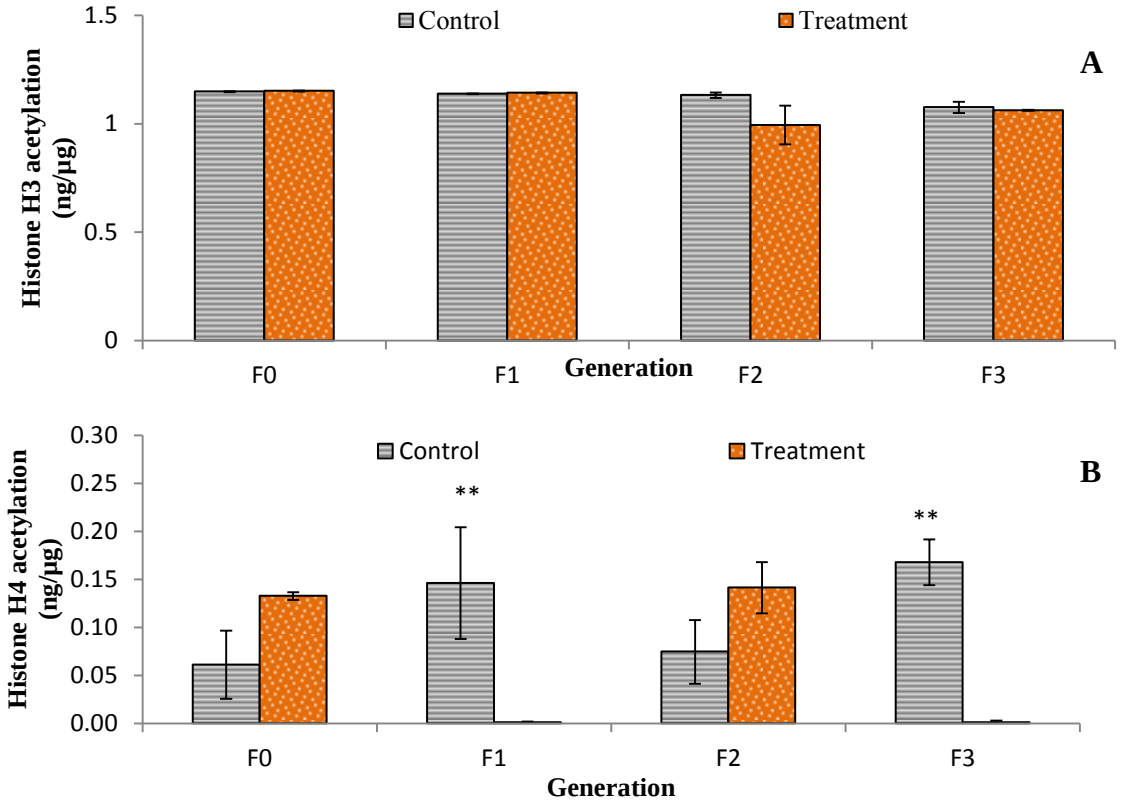

Fig. 6.8: Acetylation level of histone (A) H3 and (B) H4 in the parental *Artemia* and three successive generation progenies (common garden test). Total histone (3 µg) extracted from F0 to F3 juveniles was analyzed for total histone H3 and H4 acetylation using the Episeeker histone H3 or H4 total acetylation detection flurometric fast kit. Values are represented as means of 4 biological replicates. Asterisks represent a significant difference between the control and treatment of the respective generation * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

Vibrio campbellii*-exposed *parental generation and the 3 successive unexposed generations exhibited stochastic pattern in the trimethylation level of histone H3 at lysine 4 (H3K4me3)

To gain more insights, we next attempted to analyze histone H3K4me3 which is associated with giving rise to detectable transcription of genes (Illingworth et al. 2010; Maunakea et al. 2010). As shown in **Fig. 6.9**, the induction level of H3K4me3 was markedly lower in the T-F0 parental *Artemia* than that in the control C-F0 parental group. Relatively lower level of H3K4me3 was also detected in the T-F1 progeny compared to the C-F1control. However, at F2 generation the H3K4me3 levels increase considerably in

the T-F2 progeny, whose grandparents were exposed to *V. campbellii*, compared to the corresponding C-F2 control group. The induction level of H3K4me3 between the T-F3 and C-F3 progenies did not appear to be different. These data on H3K4me3 methylation, together with the data on histone H3 and H4 acetylation suggest that histone proteins are modified in a direct or indirect manner to control the regulation of immune-related genes and induce resistance within the host against pathogens.

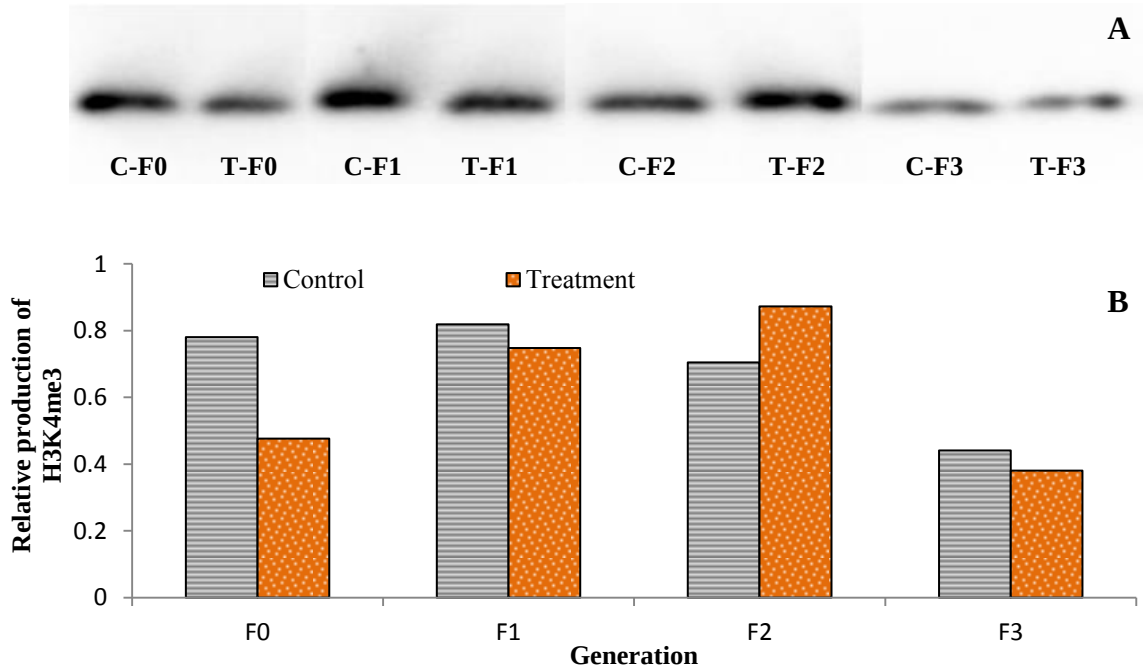

Fig. 6.9: Immunoblot analysis showing three methylation levels of histone H3 on the K4 residual tail (H3K4me3) in the parental *Artemia* and its three successive generation progenies (common garden test). (A) Total histone was extracted from the generation F0 to F3 juveniles and was resolved in SDS-PAGE gels and then either stained with Coomassie Biosafe or transferred to polyvinylidene fluoride membrane and probed with antibody that recognizes H3K4meE. *Artemia* histone protein (15 µg) was loaded in each lane. HeLa cells (6 µg) were loaded onto one well to serve as a positive control and for calculating the amount of histone H3K4me3 acetylation in the sample. Cropped blot image was shown (B). This quantitative analysis of histone H4 in *Artemia* is expressed as relative to the HeLa cells.

Discussion

The phenomenon of immune priming has been reported in multiple groups of invertebrates (Watson et al., 2005; Sadd and Schmid-Hempel, 2006; Dong et al., 2006;

Tidbury et al., 2011), but the specificity, adaptive characteristics, and the molecular basis of this phenomenon remain unclear (Hauton and Smith 2007). In this study, we address these in a more comprehensive approach by carrying out a transgenerational study, wherein a population of parental *Artemia* was immune challenged at early stages of their life with *V. campbellii* exposure, and the resistance of 3 successive, unexposed generation progenies towards the same or different bacterial strain was analyzed. Our results provided conclusive evidences suggesting for the occurrence of specific immunological protection in the invertebrate *Artemia*, as manifested by increased resistance of the (T-F1) progeny of *V. campbelli*-exposed parents towards the same bacteria their parents had been previously exposed to, rather than to unfamiliar type of bacteria (H6 *Vibrio*). Interestingly, similar to specific and adaptive immune response in vertebrates, the specific immune priming effect appeared to be long lasting in the invertebrate *Artemia* because of the persistence of the acquired protective response in the successive generation, whose ancestors were exposed to *V. campbellii*. The bacterial-resistant tests were conducted under both gnotobiotic (germ-free) and conventional conditions to verify the observed effects across generations. The gnotobiotic *Artemia* culture system was employed for such tests because it is a crucial tool that allows eliminating the possible interference of different microbial communities that exists in between the F1 to F3 generation progenies and furthermore facilitates studying the effects of interaction only between the germ-free progeny and the tested bacteria. Under both these experimental conditions, we observed similar acquired-resistance phenotypes across the T-F1 to T-F3 progenies. This is the first report to our knowledge to verify under both germ-free and conventional conditions and across multiple generations the phenomenon of 'specific transgenerational immune priming' in the invertebrates. Previous studies were conducted in conventional systems and were only till first generation progeny to verify the phenomenon of transgenerational immune resistance (Little et al. 2003; Grindstaff et al., 2006; Moret et al., 2006; Roth et al., 2010). It is important to mention here that the effects seen across the generations in our study is less likely due to selection on the parental populations. It is because, in another study, which is an extension of the present work, we observed that upon *V.*

campbellii-based immune challenge of an apomictic parthenogenetic *Artemia* population produced from a single female (i.e. a female parthenogenetic *Artemia* population clone, which has no other mechanism for genotypic change but mutation, which may induce genetic differentiation (Abatzopoulos et al., 2003), its 3 successive, naïve generation progenies exhibit similar increased resistance towards the same bacterial challenge (Chapter 4). However, it is also not unlikely that the observed phenotypic responses across the generations is due to genetic mutational event (such as bacteria-facilitated mutation; Mager, 2006) but this remains unknown and needs further investigation.

As is true for the adaptive immune response in general (Frost, 1999), generation of transgenerational immune defense in invertebrates is costly (Moret, 2006; Moreau et al., 2012) and can results in trade-offs with other life-history traits, such as reproduction and development (Schmid-Hempel, 2005; Freitak et al., 2005; Roth et al., 2010). However, our results suggested that there is no cost for immune priming of the parental generation for the net reproductive success/fitness of the progeny for 3 successive generations, suggesting that the observed specific immune priming across generations occurred without trade-off of other fitness traits. However, significant increase in cyst production in *Vibrio*-challenged animals and their successive unchallenged progenies could be considered as an adaptive response of the animals associated with stress mediated by pathogen attack.

Many signaling molecules, such as HSP70 and HMGB1 are known to be induced during (pathogenic) stress response and play important roles in defining the tolerance of organisms against this stressor (**Chapter 4**; Baruah et al., 2012; Baruah et al., 2014). These HSP70 and HMGB1 molecules are proteins that perform multifaceted functions, such as they act as molecular chaperone for protein and DNA, respectively, both function as an danger associated molecular protein (DAMP) during inflammation and various cellular processes (Tang et al., 2011; Lu et al., 2014). In presence of pathogenic biotic and abiotic stressors, extracellular HMGB1 and HSP70 participate in the activation of cell surface innate immune receptors, thereby modulating many aspects of host's immune responses (**Chapter 4**; Lotze and Tracey, 2005; Harris et al., 2012). In our study, the

observed positive correlation between elevated *hsp70* and *hmgb1* gene levels (as induced by *Vibrio*-based immune challenge of the T-F0 generation) and increased survival in each generation (T-F1 to T-F3) suggests that these signaling molecules may be involved in mediating enduring resistance to the successive generation, naive progenies of the *Vibrio*-challenged ancestors. In line with our results, a similar positive correlation was also detected in our previous study between elevated HSP70 protein levels (as induced by daily nonlethal heat shocks of the parental generation *Artemia*) and increased resistance of 3 successive generation, unexposed progenies whose ancestors were exposed at early stages of their life to non-lethal heat stress (**Chapter 3**).

The reason that naive T-F3 progeny whose ancestors were exposed to *V. campbellii* had increased resistance towards subsequent *V. campbellii* challenge despite no increased levels of *hsp70* and *hmgb1* genes is less clear, although one could argue the expression/production of other molecular chaperones, such as HSP40, HSP60 and HSP90 in this progeny may provide a partial explanation (**Chapter 4;** Baruah et al., 2014). Others (see review, Harris and Andersson, 2004; El Mezayen et al., 2007) who studied the role of HSP70 and HMBG1 protein molecules in mediating immune responses in other model systems found that both HSP70 and HMBG1 initiate inflammatory responses in many disease conditions via a receptor-mediated mechanism. The pathogen recognition receptor DSCAM is a highly plastic one that has previously been shown to mount adaptive(-like) immunity in invertebrates through pathogen specific alternative splicing during the pathogen encounters (Watson et al., 2005; Dong et al., 2006; Smith et al., 2011; Jin et al., 2013). A study on the invertebrate *Anopheles gambiae* showed that the DSCAM genome of this animal has a complex organization with 101 exons that can produce over 31,000 potential alternative splice forms with different combinations of adhesive domains and interaction specificities that can react specifically to pathogen and protect the animals against the infection (Dong et al., 2006). In another study, Watson et al. (2005) showed that *Drosophila* immune-competent cells have the potential to express more than 18,000 isoforms of the DSCAM molecules generated through alternative splicing which is highly conserved across major insect orders and has considerable impact

on the efficiency of phagocytic uptake of bacteria. Besides DSCAM, LGBP is another PRR which play crucial roles in generating innate immune defense against Gram-negative bacteria (like *V. campbellii*) (Chen et al., 2014). Several lines of evidences suggested increased levels of LGBP in presence of pathogens or pathogen associated molecules (such as LPS) in aquaculture invertebrates, such as tiger shrimp (Sritunyalucksana, et al., 2001, Amparyup et al., 2012), *Penaeus stylirostris* shrimp (Ruax et al., 2002) and crayfish (Lee et al., 2000). In our study, we did not see a clear link between the genes encoding for the *hsp70* and *hmbg1* signaling molecules and that of *dscam* and *lgbp* PRRs. However, it appears likely that *hsp70* and *hmbg1* may be associated in the generation of specific defense responses in the T-F1 to T-F3 progenies of *Vibrio*-exposed ancestors through other immunological receptors rather than through DSCAM and LGBP. Further research is required to determine the type of PRRs expressed in the T-F1 to T-F3 progenies and their downstream effect on the generation of immune responses. Previously, in a related study, Pham et al. (2007) demonstrated that the invertebrate *Drosophila melanogaster* infected with *Streptococcus pneumonia* or *Beauveria bassiana* (a natural fruit fly pathogen) are protected for reinfection with the same bacterial pathogen, but not with other pathogens, and established that these protective mechanisms during reinfection was ascribed to activation of the hemocytes through Toll receptor-mediated pathway.

Despite no significant changes in the expression levels of *dscam* and *lgbp*, a few downstream genes known to be involved in humoral innate immune response showed altered expression levels across three successive generations in response to *Vibrio* challenge of their ancestors (see **Fig. 6.7**). This results support our above-mentioned assertion for the involvement of other receptor-mediated pathways in the initiation of specific immune responses. Among the immunity-related genes, those encoding for the immune effectors *proPO, tgase* and *ftn* were upregulated in a stochastic manner across the T-F0 to T-F3 generations. However, the gene encoding for *pxn* remained elevated both in the parental generation in response to *Vibrio* exposure as well as in their successive generation, unexposed progenies. The *proPO* and *tgase* genes are the important constituents of the innate immune repertoire of invertebrates, including

Artemia, that protect the animal against invading pathogens by their role in melanization and coagulation, respectively (Cerenius et al., 2008, Gao et al., 2009; Baruah et al., 2014). The *ftn* gene encoding the protein ferritin participates in invertebrate's innate humoral response to bacterial infection by mechanism of withholding iron, an essential nutrient for growth and survival of bacterial (Kong et al., 2010). The peroxinectin-encoding gene *pxn* on the other hand is multifunctional immune component in invertebrates involved in various biological processes, such as cell adhesion, hemocyte degranulation, opsonization, encapsulation enhancement, nodule formulation, peroxidase activity and transduction pathway regulating the expression of antimicrobial peptides (Johansson and Söderhäll, 1989; Kobayashi et al., 1990, Johansson, 1999; Thornqvist et al., 1994; Cerenius and Sonderhall, 2004; Cerenius et al., 2008; Dong et al., 2009). From our results reported above, we can suggest that there is a non-linear relationship between the immunity-related genes (except for *pxn*) and the observed acquired resistance traits in the T-F1 to T-F3 generations. In invertebrates, each of the effector systems involved in the immune response may carry a different cost when activated and their relative expression may shape the cost of the whole immune response to a standard challenge. The observed variation in the degrees of expression between the effector systems involved in the immune response might be an adaptive defensive strategy of the progenies for subsequent pathogenic challenge, while minimizing the potential costs of the immune response (Moret and Siva-Jothy, 2003).

For the offspring to remember a past experience from the parents, the latter have to be able to perceive the specific stress, they have to store this information, retain and transmit it to the progenies. To benefit from this information the progenies have to be able to retrieve the information and translate it into appropriate reactions. Although the molecular mechanisms behind same-generation immune priming have recently started to be better understood (for review, see Conrath, 2011), the molecular details underlying transgenerational immune priming phenomenon still remain unknown. However, recently a few studies have commented on the possible involvement of epigenetic mechanisms in transgenerational phenomena in plant models, organisms that are also devoid of adaptive

immune system similar to invertebrates (Chinnusamy and Zhu, 2009; Alvarez et al., 2010; Sano, 2010). Histone modifications (in terms of acetylation or methylation) are examples of epigenetic mechanisms; indeed, such modifications in the promoters of defense genes have been shown to correlate with transgenerational induced resistance in *Arabidopsis* against abiotic (Boyko et al., 2010) and biotic stresses (Luna et al., 2011). In our earlier study, we have demonstrated that, on exposure to an abiotic stressor, a parental population of parthenogenetic *Artemia* experiences an increase resistance against pathogenic *V. campbellii* and this acquired resistance phenotype were transmitted to three successive generations, none of them were exposed to the parental stressor (**Chapter 5**). Results also suggested that the transgenerational inheritance of the increased resistance traits was mediated by increased acetylation states of the epigenetic marks histones H3 and H4 (**Chapter 3**). In the present study, we observed alterations in the acetylation and methylation levels of H4 and H3K4 histones, respectively, but not of histone H3, across the T-F0 to T-F3 generations; however, these epigenetic changes did not correlate with the transcription levels of immune genes analyzed as well as with the observed acquired resistance traits. As mentioned above, we have analyzed total histone acetylation/methylation levels and not immune-gene specific histone modifications (e.g., *proPO, tgase, pxn* and *ftn*). This result, therefore, does not eliminate the possibility that the observed enhanced resistance at each generation are due to epigenetic modifications at specific immune gene(s). It is also important to mention that besides functioning as signaling molecules, HMGB1 is a non-histone chromatin-associated protein, which function is to stabilize nucleosome (histone/DNA complex) formation and to act as transcription-factor like protein that regulates gene expressions by bending DNA and promoting access to transcriptional proteins on specific DNA targets (Muller et al., 2001; Park et al., 2003; Klune, et al., 2008). The association of this non-histone mark in the appropriate regulation of the immune genes, leading to the induction of specific resistance at each generation cannot be excluded. Currently, little is known on how these histone and non-histone proteins, together with DNA methylation (another epigenetics mark responsible for mediating immune priming, Slaughter et al., 2012) interact among each

other in regulating appropriately the expression of genes in the immune cells that leads to (specific) transgenarational immune priming phenomenon in the invertebrate *Artemia*. These mechanistic details need further verification.

In conclusion, in this study we provide firm evidence that innate immunity in the invertebrate *Artemia* has adaptive features and that it has the capacity to induce long lasting protective effects against subsequent challenge with the same pathogen. This process of immune priming likely represents a paradigm shift in immunity, as it demonstrates the existence of specific immunological memory in the absence of adaptive immune responses. Better insights into the role of epigenetic reprogramming of innate immunity in invertebrates (immune priming) may have important consequences for vaccine design (more specifically with regard to the selection of antigens) for shrimp aquaculture industry to combat diseases challenging this high-food value organism by naturally conferring resistance to infections.

PART 4

Discussion and Conclusions

Nothing in the world happens by accident. Prejudice in science and philosophy means walking away from the truth.

- Abu Ali Sina (980 - 1037)

CHAPTER 7

General Discussion and Conclusions

Stress, adaptive response and epigenetic mechanisms

Preserving the genome stability and integrity is the primary goal of every organism. At the same time, animal genomes are in a constant state of flux influenced by exposure to a variety of environmental stimuli (Arnholdt-Schmitt, 2004; Madlung and Comai, 2004). The animals respond to these environmental stimuli, commonly referred to as stressors, by modulating a variety of physiological and cellular processes including the mechanism of resistance and tolerance (Tort, 2011). The most common features of the physiological stress response is the rapid release of stress hormones into the circulation, in an attempt to activate a number of metabolic pathways that result in the mobilization of energy substrates to cope with stress. The cellular stress response represents the production of molecular chaperones, such as heat shock proteins (HSPs) (**Fig. 7.1**). The stress response of organisms to any exogenous or endogenous perturbation, from physiological to the molecular levels, usually facilitates the initiation of acclimation and adaptation processes (Hermans et al., 2006; Dalvi et al. 2009; Sarma et al. 2010). These include the immediate and long-term strategies of tuning metabolic processes to fit the current environmental conditions (Baena-González, 2010).

It is, however, important to mention here that there is a distinction in the response of organisms towards the types of stress i.e. to acute and chronic stress, under many different circumstances, although both types generate stress responses via different pathways (Tort, 2011) and have different repercussions on the animals (Baruah, 2012).

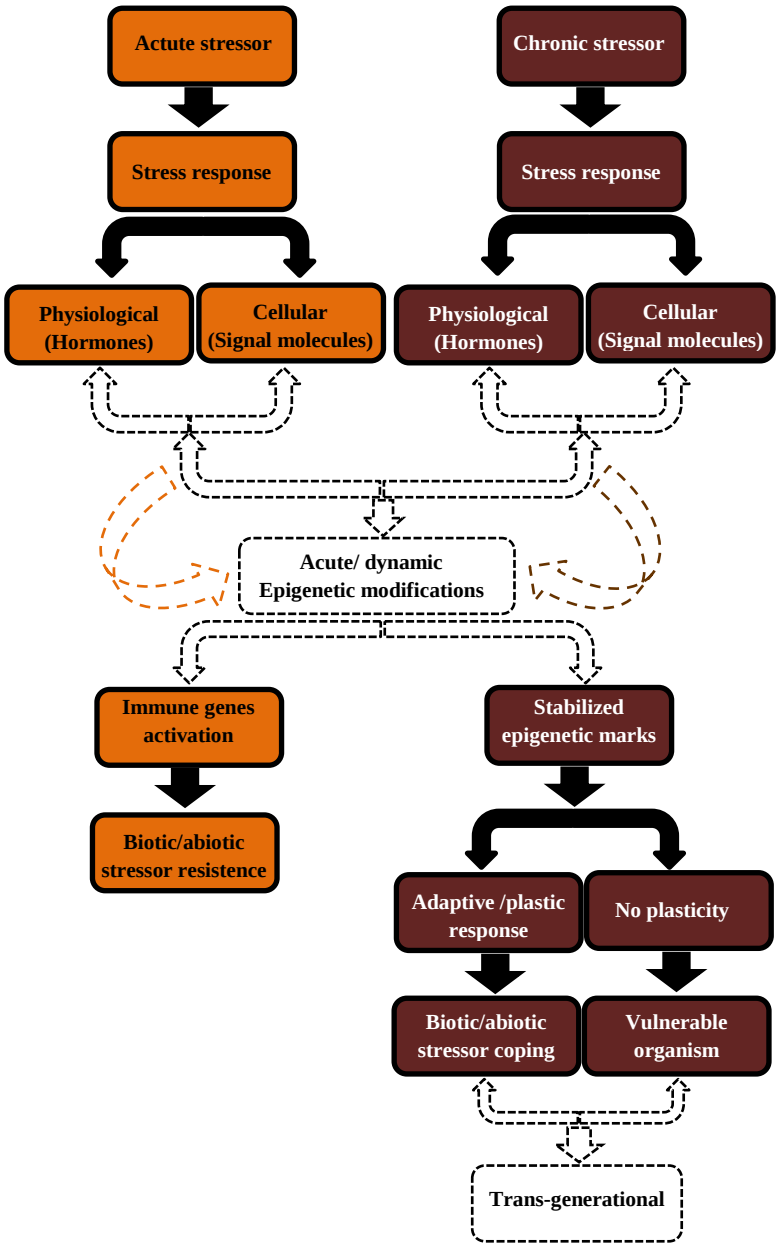

Fig. 7.1. Stressors, stress response and phenotypes in animals. This figure briefly describes how the acute or chronic stress can epigenetically influence the faith of organism robustness phenotype and that of subsequent generation or generations. Once stressed, organisms activate two independent pathways to maintain the homeostasis of the body. One pathway is physiological and involved stress hormones. The other pathway is cellular and involves signal molecules activation. Both physiological and cellular (Radtke et al., 2011; **Chapter 3**) responses are capable of modifying epigenome. These modifications can affect the hormone receptors, gene expression and phenotypes which can be trans-generational. Epigenetic modifications can further maintain or suppress the activated responses.

For example, it has been reported that acute stressors which normally last for a short term and with a high intensity (such as predation, fighting) cause a fight-or-flight, resulting in the survival of the stress episode (or the death of the individual). On the contrary, during chronic situations in which the intensity of the stressor is low but persistent, there is a switch of energetic metabolism to cope with the demands of the stressor. This involves more energy and resources allocation to the stress process resulting in increased susceptibility to stressors. Gilthead sea bream affected by the winter syndrome shows high susceptibility to *Pseudomonas anguilliseptica* infection and a more severe immune suppression is observed when compared to normal fish (Tort et al., 1998). Pathogen susceptibility in stressed catfish resulted in an increase of 20% in mortality (Small and Bilodeau, 2005).

Although stressors induce suppressive or adverse effects, part of the responses, as described above, could be also considered as active or adaptive and therefore, the resolution of this complex event depends on the time course, the induced response and the stressor (Iwama et al., 1999; Tort, 2011). In a previous study (Dhabhar, 2002), prior exposure to stress was reported to determine the ability of building a further response and therefore the efficiency of the regulatory systems, including immune activity. This indicated that earlier experience may bring an advantage to those animals when they encounter a further stressor. The phenomena of adaptive response or increased tolerance to a subsequent stressor in response to prior exposure to a same or different one have been demonstrated in various animals (Dalvi et al. 2009; Sarma et al. 2010; Sung et al., 2012). In fact, in a previous study carried out in the Lab of Aquaculture & *Artemia* Reference Center, a non-lethal heat shock (a sort of acute stress) was shown to protect *Artemia* larvae against subsequent biotic and abiotic stressors. It was observed that larvae given a preconditioning treatment at 37 °C for 30 min with 6 h recovery had higher survival when exposed to chronic exposure to pathogenic *Vibrio campbellii* challenge (Sung et al., 2007) or to abiotic lethal heat stress (Baruah et al. 2010). Similarly, a combined hypo- and hyperthermic stress followed by recovery at ambient temperature safeguarded *Artemia* larvae against subsequent challenge with pathogenic *V. campbellii*. Enhanced survival

coincided with the induction of HSP70 production (i.e. cellular stress response), suggesting that this HSP70 are directly involved in protecting larvae from the pathogenic stressor (Sung et al., 2008).

The ability of (aquatic) animals to acclimate or adapt to a new stress within a generation or across generations cannot be explained by random mutagenesis followed by the selection of the fittest individuals. As the frequency of spontaneous mutations is rather low, it would require many generations to establish and select the corresponding trait(s) in the animal population. On the contrary, as discussed above, multiple studies suggested that increased tolerance to stress could be observed both within the generation (Baruah et al. 2012; 2014) and also in the immediate progeny of parental animals that were exposed to environmental cues at the early stages of their lives (Whittle et al., 2009; Boyko et al., 2010, Kathiria et al., 2010; **Chapter 3**). However, what remain unclear yet is the possible underlying mechanisms behind such fast acclimation/adaptation.

It can be hypothesized that the transient but stable mechanisms involved in the heritable changes in gene expression and function that cannot be explained by modifications in DNA sequence named epigenetics (Bird, 2007), are responsible for these adaptive phenomena. Epigenetics is the mechanism by which the genes record or adapt to the environmental variations and can shape gene expression over a few minutes, an hour, or a lifetime (Peckham, 2013). The later one can even shape the gene expression pattern of the next generation (**Fig. 7.1**; **Chapter 3**). The exact molecular bases for these epigenetic mechanisms are slowly being unraveled. Epigenetic modifications can readily activate or completely terminate the regulation of particular genes through mechanisms of methylation of cytosine residues in the DNA, remodeling of the chromatin structure through chemical modification, and the regulatory processes mediated by small RNA molecules. These kinds of processes can act individually or in combination (Berger, 2007). The epigenetic marks enable the genes to remember the stressful event and make a plan for its possible recurrence (Peckham, 2013).

In an intensive aquaculture practices, the shrimps and other crustaceans are often subjected to thermal perturbation as well as to pathogen (vibrios) exposure, causing

significant stress to the animals. Therefore from aquaculture perspectives, abiotic heat stress and biotic *Vibrio* infection are factors of great relevance, because these two factors are the most commonly encountered environmental stressors, and additionally they are also the prime causative agents for hindering sustainable aquaculture production. In the first part of this thesis, the effects that environmental heat stressor can have on the induction of increased resistance phenotypes both within the generation and across generations in the shrimp model *Artemia* and their underpinning mechanisms were investigated.

Parthenogenetic *Artemia* as model organism for transgenerational epigenetic inheritance studies

Selection of an appropriate animal model that allows studying transgenerational epigenetic inheritance of acquired traits in response to environmental cues is of high importance. *Artemia* offers a variety of benefits that makes it an excellent model organism for epigenetic studies. Based on their mode of reproduction, *Artemia* can vary between bisexual or asexual (parthenogenesis). Parthenogenetic species of *Artemia* can produce clonal population through apomixis where all the progenies are female (for details, see review of literatures). In this mode of reproduction meiosis does not occur and therefore the chance of recombination is very low. Thus the entire populations of progenies are clone of the mother. Such reproductive behavior minimizes genetic variation between the progenies, which offers a unique opportunity to separate genetic and epigenetic influences on the phenotype, an invaluable asset when studying epigenetics. The attractiveness of (parthenogenetic) *Artemia* as a potential epigenetic model organism is further enhanced by the fact that the storability of cysts produced in each successive generation allows for 'common garden' experiments, i.e. the simultaneous testing of animals from subsequent generations, a convenient property if one seeks to minimize environmental interference in experiments. Additionally, as this animal has short generation time, high fecundity, and can be easily cultured, it allows

investigating the effect of a broad spectrum of environmental factors on the phenotypic responses for multiple generations.

Abiotic heat stress and transgenerational epigenetic inheritance of robustness phenotype

In the first experiment, using a population of parthenogenetic *Artemia* obtained from a single female, the effects of environmental heat stress on the emergence and inheritance of phenotypic traits across three subsequent non-stressed generations were investigated (**Chapter 3**). Furthermore, the mechanistic links behind the transmission of phenotypes across generations were also looked at. To address this, the parental population of parthenogenetic *Artemia* was exposed to two daily non-lethal heat shocks at early stages of their life. The first heat shock was given at 35°C for 30 min, followed by a recovery period of 75 min at 28°C. The second heat shock was applied at 38°C for another 30 min. This process was continued for 14 days ahead of the reproduction period to ensure that the uterus carrying the cysts/embryos was not directly exposed to the experimental stress conditions. The control group, cultured isothermally at 28°C, went through the same handling process. The results demonstrated that the daily hyper thermic treatment of parental generation resulted in the development of phenotypic traits related to increased tolerance against lethal heat shock and pathogenic *V. campbellii* in the immediate generation (F1) progeny. Interestingly, this increased resistance traits also persisted in the two successive, unexposed generations without diminution. Similar results were obtained when this resistance tests were repeated in a common garden experiment, an experimental conditions which allows avoiding the interference of the changing environmental conditions and microbial communities on the outcome of the results. All the parthenogenetic *Artemia* in this experiment originated from a single female and are proposed to be genetically identical. The observed effects, therefore, are most probably non-genetic in origin. Previous studies have clearly established that there exists a strong co-relation between HSP70 production level and improved resistance of *Artemia* against biotic and abiotic stressors (Sung et al., 2008; Baruah et al., 2012; Baruah et al., 2013).

The role of HSP70 in mediating improved resistance is due to the fact that it plays a crucial function as molecular chaperone, involving in protein biogenesis and protein homeostasis in the cells or it contributes to the generation of protective immune responses in the host (Clegg et al., 2000; Baruah et al., 2013). As expected, results showed that exposure of the parental *Artemia* to daily nonlethal heat shocks markedly increased HSP70 production relative to the non-heat-shocked control groups. Remarkably, this increased HSP70 level was also observed in the isothermally reared progeny for 3 subsequent generations. These results indicate a strong positive correlation between HSP70 production and improved resistance in the current study supporting the results of earlier studies (Baruah et al. 2010, 2014).

The mechanistic bases for the heritability of phenotypes, such as stress tolerance and elevated HSP70 production as observed in the current study, remain unclear. However, stable regulation of gene expression via epigenetic modification, such as histone modifications, has often been proposed as candidate mechanism. Histone acetylation is associated with chromatin relaxation and the initiation of transcription (Levenson and Sweatt, 2005; Fischer et al., 2007). Such modifications are likely to be important players in selective epigenetic tagging when environmental stressors are involved. In this study, the acetylation states of histone H4 and H3 in the parental generation increased in response to daily nonlethal heat shocks. These modifications further transmitted across the generations, coinciding with our observed findings of elevated HSP70 and increased stress tolerance in all the three successive generation progenies of nonlethal heat-shocked ancestors. These findings suggested that acetylated histone H3 and H4 in the F1 to F3 progenies of treated F0 are casually related to heat shock memory, favoring prompt binding of transcription factors to heat shock element of *hsp70* (or other *hsps*) gene, and subsequent rapid activation of the cytoprotective arsenal (Tetievsky and Horowitz, 2010, Bilichak et al., 2012)

Besides histone modifications, other mechanisms for transgenerational inheritance of resistance phenotypes may exist, and global DNA methylation process (mostly known for its gene repression activity, Aguilera et al. 2010) may be a good candidate for

involvement. It is because in a number of earlier studies, alteration in DNA methylation, in response to environmental cues, has been associated with the transmission of imprinted genes and also adaption to stress (Boyko et al., 2010; Verhoeven and van Gurp, 2012). In this study, we did not investigate the methylation pattern of specific known imprinted genes, but focused on the total genome of the animals at every generation and the results revealed that the methylation pattern of global DNA across three successive generations in response to mild heat shocks of their ancestors was stochastic i.e., highly variable. In fact, at F0, we observed a decrease in the global DNA methylation level due to mild heat shocks, this supports our assumption that hypomethylation is needed for up regulation/production of HSPs or other stress related genes and accordingly, we observed a marked increase in the HSP70 level in this F0 group, in line with other authors (Cho et al., 2012; Gan et al., 2013). Surprisingly, the F1 progenies of heat-shocked parents exhibited a markedly increased global DNA methylation. Furthermore, the F2 and F3 progenies whose ancestors were heat-shocked, the methylation level remained unaffected. But these results did not show a clear correlation with phenotypic traits that we observed in the F1 to F3 generations. As mentioned above, we have analyzed the global DNA methylation and not on specific loci (e.g., HSP70). From our current data, therefore, it cannot be established whether the observed methylation effects and observed phenotypic effects are in fact causally related. This result, however, does not eliminate the possibility that the observed phenotypes at each generation are due to DNA hypo/hyper methylation at specific gene(s). In addition, little is known on how DNA methylation and histone (H3 and H4) proteins interact among each other in regulating the epigenetic inheritance of phenotypic traits across multiple generations. Further studies are required to address these possibilities.

Abiotic heat stress and induction of a molecular chaperone HMGB1 protein

In the above study, it was unequivocally demonstrated that non-lethal heat shock treatment plays a significant role in the induction of HSP70, which subsequently contribute to the trans-generational induction of robustness. In view of the established fact that under stressful conditions, besides HSP, other molecular chaperones, such as HMGB1 protein, are released either actively or passively into the extracellular environment (Mayer and Bukau, 2005; Wang et al., 2009; Tang et al., 2011), therefore subsequent study was carried out to determine whether HMGB1 is responsive to environmental stress factors, and whether it has a downstream effect on the induction of resistance within an organism (**Chapter 4**). This study was conducted within a generation using the gnotobiotic *Artemia* test (GART) system (**Fig. 7.3**). GART provides the opportunity to test the *Artemia* under germ-free environment, and a controlled species and population of micro-organism can be added. This system is a crucial tool for such studies because it allows eliminating the interference of the microbiota that are naturally present in any type of aquatic environment and furthermore facilitates the interpretation of the results in terms of a cause effect relationship (Marques et al., 2004; Baruah et al., 2014). In this study, *Artemia* nauplii harvested from the GART system were exposed to NLHS for an indicated period and then either challenged with *V. campebellii* for a period of 12 h or not following the procedure as previously described (for methodology, see **Chapter 4**). Results showed that on exposure of *Artemia* to NLHS, the production of HSP70, both at the transcriptional and translational levels, significantly increased at 6 h, and reduced markedly to control level at 12 h post treatment. However, the HMGB1 protein showed a different production pattern in response to exposure to NLHS. In fact, at 6 h post treatment, the HMGB1 protein level did not increased significantly, however, at 12 h HMGB1 protein production increased significantly, succeeding HSP70. These results

indicate that besides HSP70, HMGB1 protein is also induced in *Artemia* in response to exposure to NLHS but in a sequential manner. Interestingly, the increased production of these two molecular chaperones corresponded well with increased survival of *Artemia* in these groups (data not shown; see Sung et al., 2008; Baruah et al., 2012), in agreement with the results of an earlier study that point towards a critical role of HMGB1 protein in conferring protection to mice against LPS–induced endotoxemia and bacterial infection by *Listeria monocytogenes* (Yanai et al., 2009). The mechanism behind the protective effects of HMGB1 in association with HSP70 is unknown yet, however, at this point it can be hypothesized that these two molecular chaperones through an unexplored cascade of biochemical and immunological reactions contributed to the protection of *Artemia* against *V. campbellii.* Further research is required to unravel detailed mechanism behind the role of these two molecules in stimulation of *Artemia* innate immunity.

Biotic *Vibrio* challenge and transgenerational immune priming phenomenon in the invertebrate *Artemia*

The crustaceans rely only on the innate arm of the defense system to fight against pathogenic biotic stressors. The innate immune system lacks the machineries to produce lymphocytes and functional immunoglobulin, the key components of the adaptive immune system in vertebrate (Fearon 1997), and hence is thought to lack the ability to show high degree of specificity and long-lasting memory (the ability to respond rapidly upon re-exposure to a particular pathogen) (Litman et al. 2005; McFall-Ngai 2007). However, a few reports have claimed that invertebrates show some form of immunological specificity and memory similar to that of adaptive immunity in vertebrates. These claims for adaptive-like immunity in invertebrates, termed ‘immune priming’, were based largely on observations of only a few species and a limited selection phenomenological observations that previous exposure to pathogens and/or immune elicitors has a positive influence on the immune system (Sadd et al. 2005; Tidbury et al.

2011) or on the resistance of the animals against subsequent microbial infections (Kurtz and Franz 2003; Sadd and Schmid-Hempel, 2006; 2007; Roth et al. 2010). Prompted by the emerging evidences (see **Chapter 4**) that in response to abiotic cues, the epigenetic marks, such as histone H3 and H4, and DNA methylations, undergo modification to regulate the expression of defense-related genes (i.e. *hsp*70) and subsequently induce inherited resistance phenotypes, the second part of the thesis aimed to determine whether immune challenge of the parental generation by exposure to pathogenic biotic stressor could induce inherited resistance phenotypes in the progenies in a similar way as observed above. In fact, more specifically, using two different strains of brine shrimp – parthenogenetic and bisexual *Artemia*, and pathogenic *V. campbellii* as host-pathogen model system, this part of the thesis aimed to unambiguously prove the possibility or impossibility of immune priming, examining the presence or absence of specific and enduring memory against homologous and heterologous pathogens (*Vibrio* spp.) during a transgenerational study, and to unravel whether epigenetic programming of the immune system is the underlying mechanism behind such phenomena (for the immune function in crustaceans, refer to **Fig. 7.2**). The results showed that upon immune challenge a clonal population of parthenogenetic *Artemia* (originating from one single female) by exposing the population at early life stages to a pathogenic biotic stressor *V. campbellii*, the three successive generation progenies (none of them were immune challenged) exhibited high resistance against subsequent challenge with the same bacterial strain (**Chapter 5**). This study was the first to provide unequivocal evidences for 'immune priming in shrimp'. As this phenomenon was observed in all the 3 successive, unexposed generations of a clonal population of parthenogenetic *Artemia*, it is therefore less likely that the observed immune priming effect in *Artemia* is due to the results from selection on the parental population. Under similar experimental approach, the above findings were further validated in a bisexual population of *Artemia*. In accordance with the above study (**Chapter 5**), the results of this study also showed increased resistance of the immediate progeny (T-F1) of *V. campbellii*-exposed parents against subsequent challenge with the same bacteria their parents had been previously exposed to, rather than against unfamiliar

type of bacteria (H6 *Vibrio*), providing conclusive evidences for the occurrence of specific immunological protection in *Artemia*. Interestingly, the specific immune priming effect also appeared to be long lasting in *Artemia* because of the persistence of the acquired protective response in the successive generation, naïve progenies, whose ancestors were exposed to *V. campbellii*. It could also be observed that improved resistance phenotypes of the progenies derived from the immune-challenged ancestors was positively correlated with elevated expression levels of the signaling molecules *hsp70* and *hmgb1*, which upon induction during (pathogenic) stress response, play important roles in defining the tolerance of organisms against this stressor (**Chapter 4**) by performing multifaceted functions, such as like molecular chaperones, DAMP during inflammation and various cellular processes, activating cell surface innate immune receptor and modulating many aspects of host's immune responses (Moret, 2006; Sadd et al., 2007; **Chapter 3)**. These results suggested that these signaling molecules may be involved in mediating long lasting resistance to the successive generation, naive progenies of the *Vibrio*-challenged ancestors. It is important to mention here that the increase in the expression of the signaling molecules in the primed animals did not lead to an increase in the expression of the *dscam* and *lgbp* PRRs. However, a number of key genes known to be involved in humoral innate immune response showed altered expression levels across three successive generations in response to *Vibrio* challenge of their ancestors. In fact, the genes encoding for the immune effectors *proPO*, *tgase* and *ftn* were upregulated in a stochastic manner across the three successive generation progenies of the immune-challenged ancestors. However, the gene encoding for *pxn* remained elevated both in the parental generation in response to *Vibrio* exposure as well as in their successive generation, unexposed progenies. This indicated that there was a variation in the degrees of expression between the effector systems involved in the immune response. This might be an adaptive defensive strategy of the progenies for subsequent pathogenic challenge, while minimizing the potential costs involved in the generation of whole-immune response (Moret 2003). The molecular mechanisms behind immune priming in shrimp remain unclear. Previous studies demonstrated that epigenetic regulatory

mechanisms (such as DNA methylation/acetylation, histone modifications) are central elements in the function of the immune system, allowing an appropriate gene expression pattern in immune cells in response to internal or external environmental cues, including pathogenic factors (see review, Fernández-Morera et al. 2010). For instance, in plant models, which do not possess adaptive defense system, epigenetic programming at the level of histone protein modifications has been proposed as the molecular mechanism responsible for long-term memory for innate immunity (Alvarez et al. 2010; Matzke and Mosher 2014) and this may also be the cause for the observed immune priming in *Artemia*. In this study, a significant alteration in the acetylation and methylation levels of H4 and H3K4me3 histones, respectively, but not of histone H3, across the progenies of the primed ancestors were observed; however, these epigenetic changes did not correlate with the transcription levels of immune genes analyzed as well as with the observed acquired resistance traits. In this study, analyses of the total histone acetylation/methylation levels and not of immune-gene specific histone modifications (e.g., *proPO, tgase, pxn* and *ftn*) were carried out. This result, however, does not eliminate the possibility that the observed enhanced resistance at each generation are due to epigenetic modifications at specific immune gene(s). At present, it is unknown how these histone and non-histone proteins, together with DNA methylation (another epigenetics mark responsible for mediating immune priming, Slaughter et al., 2012) interact among each other in regulating appropriately the expression of immunity related genes and mediating immune priming in the invertebrate *Artemia*. These mechanistic details need further verification.

The obtained results in this thesis may open new window to better understand the plasticity of invertebrates innate immune system and may provide new ways to prime commercially important aquatic animals including shrimps against biotic/abiotic stressors.

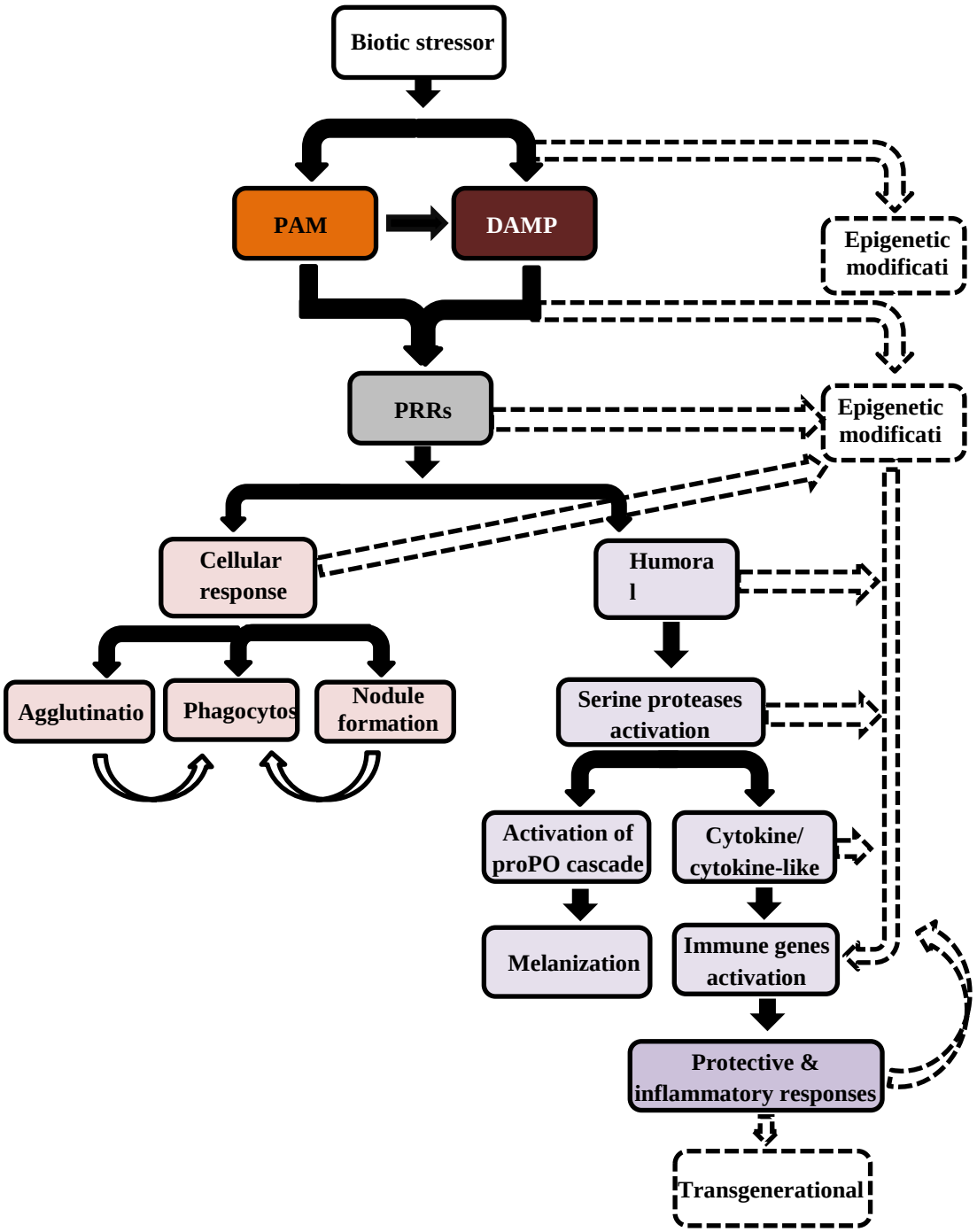

Fig. 7.2: A hypothetical model showing the mechanisms of immune priming in crustaceans. Binding of pattern recognition receptors (PRRs) to a pathogen associated molecules (PAMPs) or to damage associated molecules (DAMPs) can trigger different types of cellular (such as agglutination, phagocytosis by hemocytes) and humoral (i.e. activation of proteolytic cascades) events. These events result in the activation of the protective immune and inflammatory responses in the host. Epigenetic modification may facilitate and sustain these immune/inflammatory responses throughout the life of the host and even its subsequent generations.

Possible lines of future research

Verification of the occurrence of immune priming in Artemia under gnotobiotic experimental conditions

In the earlier immune priming experiments including the ones carried out in this thesis, the model organisms used are living in close association with several microbial communities which are naturally present in any type of environment (Baruah et al. 2014). Under such xenic (germ-associated) conditions, the model organism will get exposure to both the priming agents of interest as well as to the standing microbial communities. This indicates that under such conditions, the functional responses of the host towards the tested priming agent could simply reflect the influence of the various known and unknown microbial communities (LPS or other cellular components) rather than a genuine biological effect of the particular priming agent itself. Hence, from a mechanistic point of view, it may be unrealistic to give a logical conclusion to the outcome (i.e., specific immunity) of the priming experiments. This problem can be approached by starting with a population of axenic (germ free) animal and immune challenge this population by exposure to a known variety of bacteria. At this moment, gnotobiotic system for *Artemia* (GART system; **Fig. 7.3**) has been developed and this system can be maintained no longer than 4 days, which is too short to carry out the priming exposure of the animals. Therefore, attention should be given to develop an experimental model under gnotobiotic conditions that can be controlled over a longer period of time than the 4 days currently in the GART system and verify the immune priming phenomenon under such conditions by analyzing the resistance phenotypes of the progenies harvested from the GART system against infectious disease.

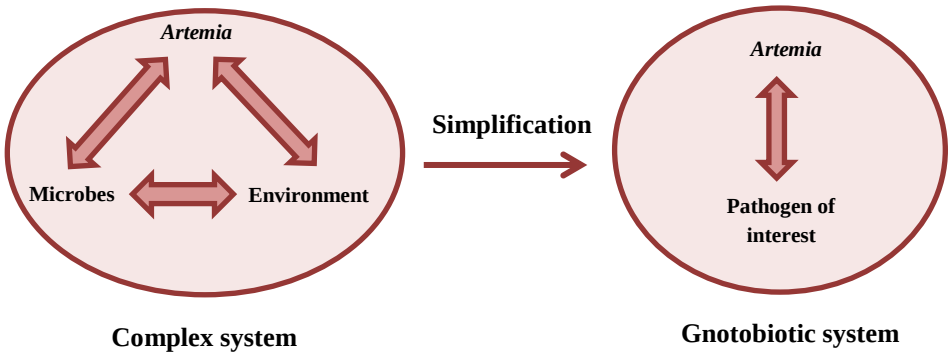

Fig. 7.3: Host pathogen interaction in a complex and gnotobiotic system.

Uncovering the mechanisms of immune priming by focusing on the immunological pathways and epigenetic programing of the immunological pathways

Unravelling the mechanism at the immunological levels

According to FAO, (shrimp) aquaculture research urgently needs to consider a more knowledge-based approach, i.e. understanding underlying mechanisms in many physiological processes, in order to be able to substantially improve production efficiencies and sustainability issues. In this thesis, evidences were provided for the occurrence of immune priming in shrimp. However, the molecular mechanisms behind such phenomenon are not very clear yet. The immune system of crustaceans has been intensively studied and there is no reason to suspect that the basic components of the immune system in *Artemia* should be different. At this moment, a panel of genes that are expected to be crucial in immune signaling in crustaceans (i.e. heat shock proteins, high mobility group box 1 protein), pattern recognition receptors (such as DSCAM, LGBP, TLRs, C1q-binding protein, and masquerade-like protein) and downstream innate humoral effector molecules (such as prophenoloxidase, transglutaminase, peroxinectin and ferritin) were known for *Artemia*. It could be of interest to examine the expression of these genes in the animals harvested from GART system as described above and their mechanistic links with phenotypic traits are established. It is also conceivable that

additional genes, besides the one mentioned above are related to immune priming in shrimp. Using the whole genome *Artemia* sequence (publicly unavailable yet) that has recently been annotated by an UGent-*Artemia* Reference Center effort, advanced molecular tools like RNA-seq technique (Kimbrel et al., 2011) should be employed to identify those genes.

It is important to mention that immune responses are regulated at translational or post-translational level. Basically, it is the functional immune proteins/enzymes that contribute to the host resistance against pathogens (**Chapter 4**). Therefore further immunoblotting and *in situ* immunohistochemistry assay should be performed on the primed animals using purpose-made commercial antibodies to validate the links. Additionally, attention should also be given on the analysis of alternative splicing of DSCAM as it has been reported as the primary mediator of immune priming in crustaceans (Smith et al., 2011; Jin et al., 2013; Riddell et al. 2014). By this, relationships between immune effector molecules and acquired resistance traits can be established.

Unravelling the mechanism at the epigenetic levels

If immune priming effects, in terms of increased immune responses were observed, a more in-depth approach should be followed to elucidate the molecular pathways involved. For that, the transcribed/translated immune effector molecules should be selected and the epigenetic changes, at the levels of DNA methylation, specific histone tails acetylation/deacetylation, in the promoter regions of these immune genes should be investigated by employing bisulfite sequencing and chromatin immunoprecipitation assays. These state-of-the art techniques will allow a very detailed analyses contributing to the elucidation of the underlying epigenetic mechanism behind immune priming in shrimp. Additionally, with the availability of *Artemia* genome, advanced OMIC tools, such as RNAi technology, micro array analysis, RNA sequencing, proteomics, whole genome sequencing, ChIP sequencing and methylation sequencing analyses should be employed to obtain a better insight into the mechanism behind the possible observed effects.

PART 5

Appendices

Nothing in life is to be feared, it is only to be understood. One never notices what has been done; one can only see what remains to be done.

- Marie Skłodowska-Curie (1867 - 1934)

APPENDIX A

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APPENDIX B

Summary

Epigenetics is the study of reversible changes in gene function that occur without modifications in the DNA sequence. The epigenome of an organism records a variety of behavioral, social, and abiotic and biotic environmental cues, providing an interface between the environment and the genome. Epigenetic variation that are environmentally determined, contributes to inter-individual variation in gene expression and thus to variation in development of phenotypes in an individual or even throughout the entire population. These epigenetic modifications can in some cases be inherited by subsequent generations, even when the initial external stimuli is no longer present in the environment of these organisms. Epigenetic modification occurs through different molecular mechanisms including non-coding small RNA molecules, DNA methylation or histone tail modifications (for details, see **Chapters 1 and 2**).

Adaptation to new environmental circumstances is the ultimate result of epigenetic modification. Among the environmental stressors animals, be it aquatic or terrestrial can encounter, pathogen attack and heat stress are quite common. In the first part of this study, by using a population of parthenogenetic *Artemia* obtained from a single female, we investigated the effects that environmental heat stressor can have on the emergence and transgenerational inheritance of phenotypic traits in *Artemia*. After daily exposure of a parental population of parthenogenetic *Artemia* to non-lethal heat shocks at early life stages, the phenotypic and epigenetic changes in the treated population and in three subsequent (untreated) generations were assessed. It was found that, on exposure to non-lethal heat shocks, parental *Artemia* experience an increase in the levels of HSP70. This acquired trait was transmitted to three successive generations, none of which was exposed

to the parental stressor resulting in their increased 1) tolerance towards lethal heat stress 2) resistance against pathogenic *Vibrio campbellii*. The transgenerational inheritance of these acquired phenotypes was associated with significantly altered levels of global DNA methylation and acetylated histones H3 and H4 in the treatment group compared to a control group, where both the parental and its successive generations were reared at standard temperature. This study was further extended to elaborate if apart from HSP70, another signal molecule is induced within one generation when animals are exposed to environmental heat stress.. In view of the established fact that high mobility group box 1 (HMBG1) protein is an intracellular molecular chaperone for DNA, and it is released into the extracellular medium upon cellular stress, similar to HSP70, for maintaining homeostatis, a subsequent study was carried out to determine whether HMGB1 is responsive to environmental stress factors, and whether it has a downstream effect on the induction of resistance within an organism (chapter 4). In this study the induction of these two proteins in *Artemia franciscana* was determined both at the levels of mRNA and protein in response to different factors (i.e. mild heat stress, pathogenic *V. campbellii*, combination of both stimuli). Results showed that in response to non-lethal heat stress, the levels of HSP70 and HMGB1 are elevated sequentially and the significant alterations in the levels of these two proteins were associated with increased protection of *Artemia* against pathogenic *V. campbellii*.

In an environment apart from abiotic stressors, animals encounter different microbial community. Some of these microorganisms are beneficial and part of them is considered as pathogens. The vertebrates respond to these pathogens using both the adaptive and innate arms of the immune system. However, the invertebrates, like brine shrimp *Artemia*, are believed to rely only on their innate immune system, which doesn't exhibit specificity and memory, to fight against invading pathogens. However, some studies reported the occurrence of adaptive-like immunity in invertebrates, phenomenon termed as immune priming and is broadly defined as increased protection to a pathogen following previous exposure to the same pathogen or an immune elicitor. This immune priming phenomenon has been suggested to be transgenerational i.e. primed parents bestow their next

generation progenies with increased resistance against subsequent challenge with same or different pathogen. In spite of these phenomenological evidences, the molecular mechanisms behind such transgenerational immune priming in invertebrates remained elusive. In the subsequent study, evidences suggesting that priming parental *Artemia* by exposing to pathogenic *Vibrio campbellii* protects their progenies against the same bacteria for three subsequent generations, but not against another strain of *Vibrio* (H6) were provided. Interestingly this increased resistance in three subsequent generation progenies against *V. campbellii* was positively correlated with elevated levels of the signaling molecules *hsp70* and *hmgb1* mRNA, suggesting the role of these molecules in mediating enduring resistance to *V. campbellii* challenge. No linear relationship between the immunity-related genes, except for peroxinectin immune gene, and the observed acquired resistance traits in the three successive generation progenies, whose ancestors were immune-challenged were observed. Furthermore, results also indicated that the levels of histone H4 acetylation and H3K4Me3 are altered transgenerationally in the group whose ancestors were primed. However, no mechanistic links were established among the observed epigenetic modifications, immune-related genes and acquired resistance phenotypes.

In conclusion, the work presented in this thesis indicated that, the organisms respond to biotic or abiotic environmental stressors with battery of adaptive phenotypes (i.e. increased resistance, different gene expression patterns and protein production). Interestingly, overall results leave no doubt on the role of epigenetic modifications, such as DNA methylation, histone H3 and H4 acetylation and H3K4Me3 on emergence of these new phenotypes.

APPENDIX C

Supplementary Materials

Our previous studies suggested that pretreatment of *A. franciscana* nauplii with a non-lethal heat shock (37 °C for 30 min), followed by a recovery period of 6 h, results in enhanced resistance of the animals against pathogenic *V. campbellii* or lethal heat shock (Sung et al., 2008; Baruah et al., 2012). This increased resistance was associated with elevated amount of HSP70 production. In a subsequent study, using parthenogenetic *Artemia* clonal population, collected from Greece (Megalon Embolon; ARC code 1279a), Vinh (2010) aimed at determining whether the resistant phenotype induced upon exposure of the animals to non-lethal heat shock is heritable. In this experiment, the animals were exposed to different regimes of daily non-lethal heat shocks from day 1 post hatching. The treatments were control: no heat shock treatment, constant ambient temperature of 28 °C; 1: one heat shock of 35 °C for 1 h; 2: two heat shocks of 35 °C for 30 min with 75 min intervals at 28 °C; 3: two heat shocks with the first one being at 35 °C for 30 min followed by a recovery at 28 °C for 75 min and the second one at 38 °C for 30 min. As readouts, the length (parental generation) and survival (F1 generation upon challenge with *V. campbellii*) of the animals as indicated below were measured at regular time period. Results showed that the length of the adult *Artemia* that were exposed to daily non-lethal heat shocks were relatively smaller than that of the control adults, which were grown isothermally (**Fig. C.1**).

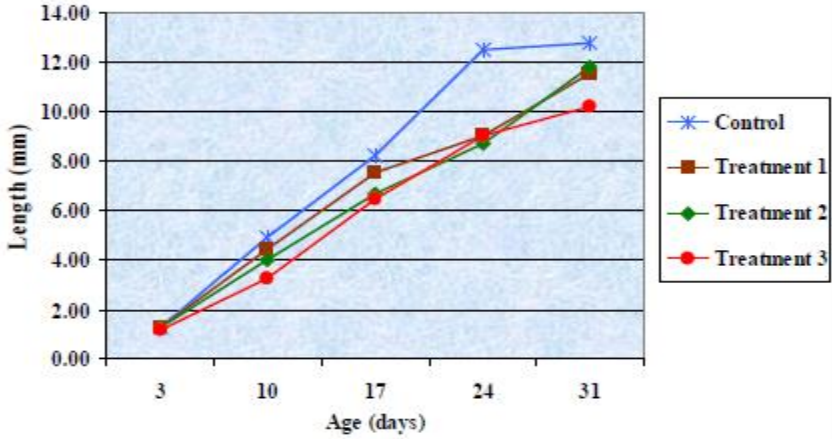

Fig. C.1: Growth (in mm) of *Artemia* during the treatment period. For explanation of the different treatments, please refer to the above text.

Once the parental generation animals were adult, they produced F1 generation naupli (ovoviviparously). The instar II naupli were collected and their resistant phenotype were measured by challenging the nauplii with pathogenic *V. campbellii* at 10^7 cells/mL (**Fig C.2**).

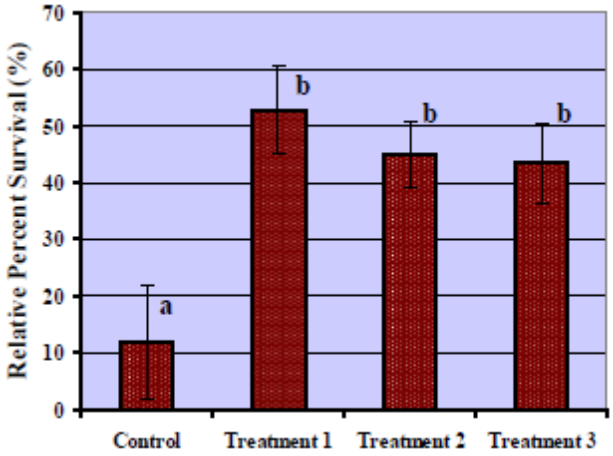

Fig. C.2: Relative survival (%) of the F1 nauplii challenged with *V. campbellii*. For explanation of the different treatments, please refer to the above text.

Results showed that the survival of the F1 generation nauplii whose parents were exposed to different treatments was significantly higher than that of the control, suggesting that F1 nauplii whose parents were exposed to heat shock treatment were more resistant compared to the control nauplii.

The above survival assay was repeated using the F1 generation cysts collected from parents treated with different treatments. However, only control and treatment 3 animals produced sufficient cysts for this experiment. Therefore, all the other treatments were excluded. Oviparously produced F1 generation nauplii collected from control and treatment 3 were either challenged with a lethal heat shock of 41 °C for 30 min (**Fig. C.3**) or with pathogenic *V. campbellii* at 10^7 cells/mL (**Fig. C.4**).

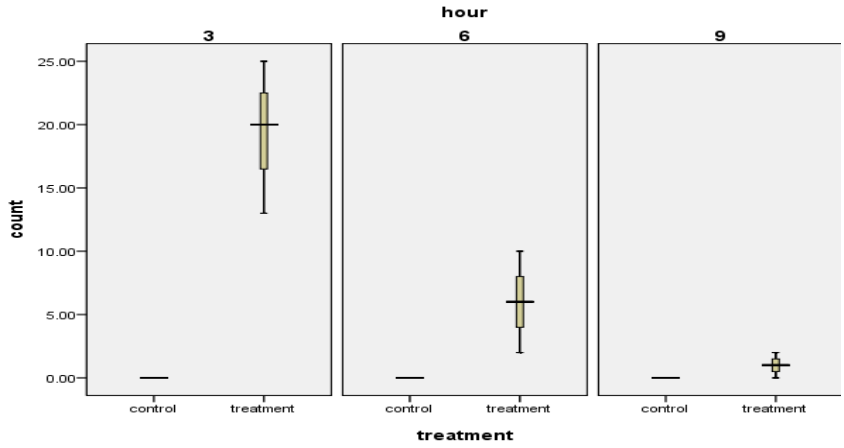

Fig. C.3: Estimation of survival for the F1 generation *Artemia* larvae challenged with lethal heat shock. The oviparous larvae (instar II), collected from parents which were exposed daily to two non-lethal heat shocks with the first one being at 35 °C for 30 min followed by a recovery at 28 °C for 75 min and the second one at 38 °C for 30 min or grown under isothermic (control) condition, were challenged with a lethal heat shock of 41 °C for 30 min. Survival was scored at 3, 6 and 9 h post challenge.

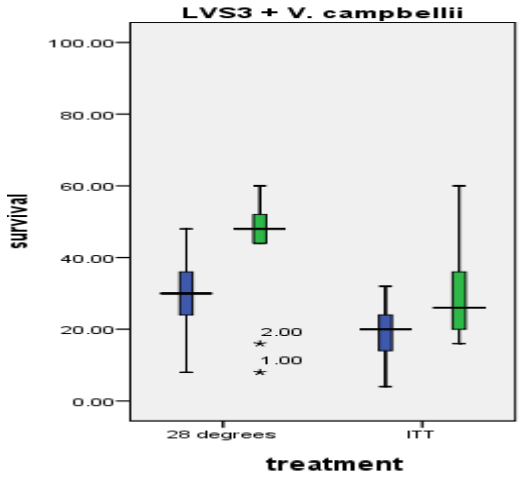

Fig. C.4: Estimation of survival for the F1 generation *Artemia* larvae challenged with pathogenic *V. campbellii*. The oviparous larvae (instar II), collected from parents which were exposed daily to two non-lethal heat shocks with the first one being at 35 °C for 30 min followed by a recovery at 28 °C for 75 min and the second one at 38 °C for 30 min or

grown under isothermic (control) condition, were fed with dead *Aeromonas hydrophila* and challenged with pathogenic *V. campbellii* (both at 10^7 cells/mL). Survival was scored at 24 h post challenge.

The results from the lethal heat shock (**Fig. C.3**) and *Vibrio* challenge (**Fig. C.4**) assays suggested that due to exposure of the parental generation with non-lethal heat shocks, the F1 generation offspring induces resistance towards lethal heat shock (abiotic) and *V. campbellii* (biotic) stressors.

The findings in the above experiments were the basis for the experiment described in **Chapter 3**. These preliminary results were confirmed, by reproducing the experiment starting from the non-lethal heat shock treatment of the parental population. However, the challenge tests (**Chapter 3**) with subsequent generations were carried out slightly different compared to the experiments mentioned above.

APPENDIX D

Curriculum Vitae

PERSONAL INFORMATION

Name	**Parisa Norouzitallab**
Date of birth	**18th February 1985**
Nationality	**Iranian**
Gender	**Female**
Marital status	**Married**

CONTACT INFORMATION

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EDUCATIONAL QUALIFICATIONS

Education	Field	Institute	Subjects	Graduation/Grade
PhD	Applied Biological Science	**Ghent University, Belgium**	Aquaculture, Epigenetics, Immunology	**By April, 2015**
MSc	Applied Biological Science	**Ghent University, Belgium**	All Aquaculture Subjects (Specialized in Fish Microbiology and Immunology)	**2010 Great Distinction (Topper)**
BSc	Environmental Resource Engineering - Fisheries	**Tehran University, Iran**	All Fisheries and Environmental Resource Engineering Courses	**2007 Great Distinction**
Pre-University	Science	**Nedaye Fatemeh School, Tehran, Iran**	Mathematics, Biology, Physics, Chemistry, Geology	**2003 Great Distinction**

THESIS TOPICS

PhD Epigenetic control of phenotypes relevant for aquaculture species using *Artemia* as model organism.

MSc Effect of Dnak mutants on *Artemia* protection in *Vibrio* challenge assay.

BSc Interaction of vitamin C & E on growth performance, stress resistance and survival of angel fish larvae.

EDUCATIONAL GRANTS

PhD BOF (Bijzonder Onderzoeksfonds) Scholarship, Ghent University, Belgium.

MSc Ghent University Master Scholarship, Belgium.

AWARDS

1. Epiconcept Conference 2013 - Best Presentation Award

Title **Epigenetic control of phenotypes relevant for aquatic species.**
Venue *Sant Feliu de Guíxols, Spain, 13-16 October*

2. Aqua 2012 Conference – Best Spotlight Presentation Award

Title **Pretreatment of *Artemia franciscana* with a heat shock protein inducer protects against pathogenic *Vibrio campbellii*.**
Venue Prague, Czech Republic, 1-5 th September

3. Aqua 2012 Conference – Best Abstract Award

Title **Pretreatment of *Artemia franciscana* with a heat shock protein inducer protects against pathogenic *Vibrio campbellii.***
Venue Prague, Czech Republic, 1-5 th September

4. Alltech – 2010 – Young Scientist Award (Local Phase Graduate)

Title **Turning carnivorous fish into vegetarians: role of phytase and acidifiers.**
Award **medal winner**

5. Alltech 2008 - Young Scientist Award (Europe & Africa Region)

Title **Natural wide spectrum anti-infective strategy for combating disease in aquaculture: a prime necessity.**
Venue http://www.alltech.com/yas/info_ea.cfm

RESEARCH PROJECT ASSOCIATED WITH

Title Aquatic systems under multiple stresses: a new paradigm integrating aquaculture and ecotoxicology research (**AquaStress**).
https://sites.google.com/site/aquastressproject/partners
Main investigator Prof. Dr. ir. Peter Bossier & Prof. Dr. Patrick Sorgeloos
Period 2012 - 2017
Budget € 3 091 513
Funding source Belgian Science Policy Office (**Belspo**)

Title *Artemia* as a model system for molecular breeding in crustacean (**blue gene**)
Main investigator Prof. Dr. Marnik Vuylsteke
Period 2012

Budget € 150 000
Funding source Industrieel Onderzoeksfonds (**IOF**), Ghent University, Belgium

MSc STUDENTS (UNDERGOING)
2014 - 2015

Name **MdSarowerE Mahfuj (Bangladesh)**
Degree MSc in Aquaculture, Faculty of Bio-engineering, Ghent University, Belgium.
Title Heat shock protein inducing compounds as preventive and therapeutic modalities against EMS disease

Name **Sarath Kumara (Sri Lanka)**
Degree MSc in Aquaculture, Faculty of Bio-engineering, Ghent University, Belgium.
Title Heat shock protein inducing compounds as preventive and therapeutic modalities against EMS disease.

MSc STUDENTS SUPERVISED (GRADUATED)
2013 – 2014

Name **Venny Mziri Mwainge (Kenya)**
Degree MSc in Aquaculture, Faculty of Bio-engineering, Ghent University, Belgium.
Title Putative mechanisms underlying heat shock protein induction in *Artemia franciscana* by a heat shock protein inducing compound.

Name **Bikila Teshome (Ethiopia)**
Degree MSc in Aquaculture, Faculty of Bio-engineering, Ghent University, Belgium.
Title Protective and therapeutic effects of compounds inducing heat shock proteins against Vibriosis.

Name **Farhana Ahmed (Bangladesh)**
Degree MSc Science and Bio-engineering Sciences, Vrije University of Brussels, Belgium.
Title Feeding ecology study of the fate of poly-β-hydroxybutyrate (PHB) as potential biological antibiotic for marine crustaceans.

2012 – 2013

Name **Ho Phong (Vietnam)**
Degree MSc in Aquaculture, Faculty of Bio-engineering, Ghent University, Belgium.
Title Effect of heat shock protein inducing compounds on the protection of *Artemia* against biotic and abiotic stressors.

Name **Priyanka Biswas (Bangladesh)**
Degree MSc in Aquaculture, Faculty of Bio-engineering, Ghent University, Belgium.
Title Effect of pretreatment of *Artemia* parental generation on the resistance of offspring against biotic and abiotic stressors.

Nam	**Linayati Linayati (Philippines)**
Degree	MSc in Aquaculture, Faculty of Bio-engineering, Ghent University, Belgium.
Title	Protective effect of compounds inducing heat shock protein in *Artemia* against *Vibrio harveyi.*

2011 – 2012

Name	**Anne Munanie Maundu (Kenya)**
Degree	MSc in Aquaculture, Faculty of Bio-engineering, Ghent University, Belgium.
Title	Pre-treatment of *Artemia* parental generation to increase the resistance of offspring against biotic and abiotic stressors

A1 PUBLICATIONS

First Author/ Equal Contribution

2015

1. **Norouzitallab, P**., Biswas, P., Baruah, K & Bossier, P. (2014). Multigenerational immune priming in an invertebrate parthenogenetic *Artemia* to a pathogenic *Vibrio campbellii*. *Fish & Shellfish Immunology.* 42: 2: 426 - 429. ***IF: 3.04***

2. **Norouzitallab, P.,** Baruah, K., Muthappa, D. M & Bossier P. (2014). Non-lethal heat shock induces HSP70 and HMGB1 protein production sequentially to protect *Artemia franciscana* against *Vibrio campbellii. Fish & Shellfish Immunology.* 42: 2: 395 – 399. ***IF: 3.04***

2014

3. **Norouzitallab, P.,** Baruah, K ., Vandegehuchte, M., Van Stappen, G., Catania, F., Vanden Bussch, J., Vanhaecke, L., Sorgeloos, P & Bossier, P. (2014). Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model. *FASEB journal*. 28: 8: 3552-3563. ***IF: 5.48***

4. Baruah, K., **Norouzitallab, P.,** Linayati, L., Sorgeloos, P & Bossier, P. (2014). Reactive oxygen species generated by a heat shock protein (Hsp) inducing product contributes to Hsp70 production and Hsp70-mediated protective immunity in *Artemia franciscana* against pathogenic vibrios. *Developmental & Comparative Immunology*. 46: 2: 470 – 479. ***IF: 3.67***

5. Niu, Y. F. **Norouzitallab, P.,** Baruah, K., Dong, S. L & Bossier, P. (2014). A plant-based heat shock protein inducing compound modulates host-pathogen interactions between *Artemia franciscana* and *Vibrio campbellii. Aquaculture.* 430: 120 – 127. ***IF: 2.59***

2013

6. Baruah, K., **Norouzitallab, P.,** Li, S. H., Sorgeloos, P & Bossier, P. (2013). Feeding truncated heat shock protein 70s protect *Artemia franciscana* against virulent *Vibrio campbellii* challenge. *Fish & Shellfish Immunology.* 34: 1: 183 – 191. ***IF: 3.04***

2012

7. Baruah, K., **Norouzitallab, P.,** Roberts, R. J. Sorgeloos, P & Bossier, P. (2012). A novel heat-shock protein inducer triggers heat shock protein 70 production and protects *Artemia franciscana* nauplii against abiotic stressors. *Aquaculture.* 334: 152 – 158. ***IF: 2.59***

2009

8. **Norouzitallab, P.,** Farhangi, M., Babapour, M., Rahimi, R., Sinha, A. K., Baruah, K. (2009). Comparing the efficacy of dietary alpha-tocopherol with that of dl-alpha-tocopheryl acetate, both either alone or in combination with ascorbic acid, on growth and stress resistance of angelfish, *Pterophylum scalare*, juveniles. *Aquaculture International.* 17: 3: 207 – 2016. ***IF: 1.04***

Other A1 Publications

2015

9. Baruah, K. Huy, T.T., **Norouzitallab, P.,** Niu, Y., Gupta, S.K., De Schryver, P & Bossier, P. (2015). Probing the protective mechanism of poly-ß-hydroxybutyrate against vibriosis by using gnotobiotic *Artemia franciscana* and *Vibrio campbellii* as host-pathogen model. *Scientific Reports* (**Accepted**). ***IF: 5.078***

2013

10. Yengkokpam, S., Debnath, D., Pal, A. K. Sahu, N. P. Jain, K. K. **Norouzitallab, P** & Baruah, K. (2013). Short-term periodic feed deprivation in *Labeo rohita* fingerlings: Effect on the activities of digestive, metabolic and anti-oxidative enzymes*. Aquaculture. 412: 186 – 192.* ***IF: 2.59***

11. Baruah, K., Nan, B., **Norouzitallab, P.,** Dierckens, K., Galindo-Villegas, J & Bossier, P. (2013). Priming in host-pathogen interactions. *Fish & Shellfish Immunology*. 34: 6: 1638-1638**.** ***IF: 3.04***

2010

12. Rahimi, R. Farhangi, M., Amiri, B. M., Rezaie, F., **Norouzitallab P &** Afzali, A. (2010). Compensatory growth assessment by plasma IGF-I hormone measurement and growth performance in rainbow trout (*Oncorhynchus mykiss*). *African Journal of Biotechnology.* 9: 25: 3949-3954. ***IF: 0.57***

2009

13. Baruah, K., Pal, A. K., Sahu, N. P., Debnath, D., Yengkokpam, S., **Norouzitallab, P** & Sorgeloos, P. (2009). Dietary Crude Protein, Citric Acid and Microbial Phytase Interacts to Influence the Hemato-Immunological Parameters of Rohu, *Labeo Rohita*, Juveniles. *Journal of the World Aquaculture Society. **IF: 0.93***

FEATURED ARTICLES

2011

1. Baruah, K., **Norouzitallab, P.** & Pal, A.K. (2011). Development of Nutrient Delivery System: A Prime Necessity for Success in Aquaculture. *European Aquaculture Magazine.* 36(2): 25-28.
2. Baruah, K., **Norouzitallab, P.** & Bossier, P. (2011). Biofloc Technology: An Ideal Approach towards Sustainable Aquaculture. *Aquaculture Europe Magazine*. 36 (3): 20-23.

2009

3. Baruah, K., **Norouzitallab, P.,** Bossier, P. & Sorgeloos, P. (2009). Quorum sensing disruption: A novel approach to combat disease in aquaculture. *World Aquaculture Magazine.* 40 (4):14-16.

2008

4. Baruah, K., Debnath, D., **Norouzitallab, P.,** Pal, A.K. & Sahu, N.P. (2008). Organic acids: The future non-antibiotic nutraceuticals in fish and prawn feed. *Aquaculture Health International Magazine* 12: 4-6.

2006

5. Baruah, K., **Norouzitallab, P.** & Sorgeloos, P. (2006). Seaweeds: An ideal component for wastewater treatment for use in aquaculture. *Aquaculture Europe Magazine.* 37 (4): 3-6.

PAPERS PRESENTED IN SYMPOSIUM/WORKSHOP/CONFERENCE

First Author

2014

1. **Norouzitallab, P.,** Baruah, K., Vandegehuchte, M., Van Stappen, G., Catania, F., Sorgeloos, P. & Bossier, P. (2014). Environmental heat stress induces epigenetic inheritance of robustness in *Artemia* model. *BIS Annual Meeting* 2014. Immunology of zoonotic infections in animal models, September 19th 2014. Ghent, Belgium (**Poster presentation**).

2. **Norouzitallab, P.,** Baruah, K., Van Stappen, G., Vuylsteke, M., De Vos, S., Sorgeloos, P & Bossier, P. (2014). Environmental heat stress induces epigenetic inheritance of robustness in *Artemia* model. Epiconcept conference 2014. Epigenetics and periconception environment. 1 – 3 October, Vilamoura, Portugal (**Oral presentation**).

2013

3. **Norouzitallab, P.,** Baruah, K., Vandegehuchte, M., Van Stappen, G., Catania, F., Sorgeloos, P. & Bossier, P. (2013). Epigenetic control of phenotypes relevant for aquaculture species. *Epiconcept Workshop* 2013. Epigenetics for Improved Food Production: from Model to Practice, 13-16 October 2013, Sant Feliu de Guíxols, Spain (**Oral presentation**).

4. **Norouzitallab, P.,** Baruah, K., Vandegehuchte, M., Van Stappen, G., Catania, F., Sorgeloos, P. & Bossier, P. (2013). Brine shrimp (*Artemia*) as an emerging model organism for epigenetic studies in aquaculture. Larvi 2013 - 6th Fish & Shellfish Larviculture Symposium Ghent University, Belgium 2-5 September 2013 (**Oral Presentation**).

2012

5. **Norouzitallab, P.,** Baruah, K., Sorgeloos, P. & Bossier, P. (2012). Protective Effect of Truncated Heat Shock Protein 70s on *Artemia franciscana* Against Pathogenic *Vibrio campbellii* challenge. Larvi 2012: The First International Conference on Larviculture, Tehran, Iran, organized by the Iranian Aquaculture Society, 11-12 December, 2012 (**Oral Presentation**).
6. **Norouzitallab, P.,** Baruah, K., Sorgeloos, P. & Bossier, P. (2012). Pretreatment of Artemia franciscana with a heat shock protein inducer protects against pathogenic Vibrio campbellii. AQUA 2012 (World Aquaculture Conference). Prague, Czech Republic, September 1- 5, 2012 (**Oral Presentation**).

7. **Norouzitallab, P.,** Baruah, K., Sorgeloos, P. & Bossier, P. (2012). An heat shock protein-inducing compound, Tex-OE®, protects *Artemia franciscana* nauplii against *Vibrio campbellii* by eliciting the innate immune system. Workshop on microbial community management in aquaculture. August 20-22, 2012, Ghent University, Ghent, Belgium (**Poster Presentation**).

2011

8. **Norouzitallab, P.,** Baruah, K., Sorgeloos, P. & Bossier, P. (2011). Feeding truncated heat shock protein 70s protect *Artemia franciscana* against virulent *Vibrio campbellii* challenge. Aquaculture Europe 2011 Conference. October 18 - 21, 2011 - Rhodes, Greece (**Oral Presentation**).

2008

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APPENDIX E

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