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Characterization and Functional Role of Heat Shock Protein 26 in *Artemia*

[1]*Faruque Miah,* [2]*Kawser Ahmed and* [3]*M.M.A. Quddus*

[1]Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Belgium
[2]Department of Fisheries, University of Dhaka, Bangladesh
[3]Department of Zoology, University of Dhaka, Bangladesh

Abstract: *Artemia*, mostly used in fish and shellfish larviculture, is a cosmopolitan crustacean living in stressful hyper saline environments. The survival of this animal is affected by different factors such as salinity, temperature, ionic composition and biotic interaction, etc. It is evident that heat shock genes and their products can play an important role in the physiology, ecology and evolutionary process of *Artemia* populations. The heat shock protein 26 of *Artemia* is one of the important heat shock protein which is directly involved to development of cyst, diapauses, quiescence and embryo and to survive larval and adult stage in different harsh environment. HSP26 is a small heat shock/α-crystallin protein composed of polypeptide subunits of 26kDa. The heat shock protein 26 transcript is undetectable in unstressed cells, but is strongly induced by heat shock, salt shock, cell cycle arrest, nitrogen starvation, carbon starvation, oxidative stress and low pH in *Artemia*. In this review characterization of heat shock protein 26 and its function in different life stages in *Artemia* has been discussed.

Key words:

INTRODUCTION

The brine shrimp *Artemia* (Crustacea: Anostraca) is normally restricted to saline inland lakes and coastal salterns [1]. In common with other large branchiopods, the brine shrimp *Artemia* is capable of producing diapausing cysts that can withstand adverse conditions such as anoxia, drying, freezing, mechanical disturbance and digestive enzymes and these cysts are therefore believed to be very suited for passive dispersal by wind, waterfowl or man [2].

Since 1950s *A. franciscana* cysts have been commercially used for aquaculture and since the mid 1990s various *Artemia* species and strains including a variety of parthenogenetic populations have been introduced to the world cyst market [3]. Another economic value of *Artemia* is link to salt production maximization, effluent treatments and cyst based toxicity test. Bio-encapsulation of non-selective filter feeder *Artemia* nauplii has been used to fulfill the nutrient requirements fish and shrimp larvae [4].

The nauplii contain abundant proteins and fatty acids are not only suitable feed for aquaculture, but are also used as favorable experimental models. Both due to the economic importance of *Artemia* and its critical role in the larviculture of fish and shellfish that, in recent years, there has been a world wide effort to discover new *Artemia* strains with specific responses to environmental conditions and to characterize them with regard to their potential use in aquaculture. Therefore, the characterization of *Artemia* populations and/or species has been a continuous endeavor since the second half of the previous century [5].

The brine shrimp, *Artemia* are found in a variety of harsh environments worldwide whereas they are exposed to severe hypersalinity, high doses of ultraviolet radiation, widely fluctuating oxygen extensions and extremes of temperature, etc. The heat shock responses are observed in early developmental stages, the encysted gastrula embryos (cysts) and nauplius larvae. Although the thermal response of these early developmental stages has been examined in reasonable detail, virtually very few are known about the heat shock response of adults including heat shock proteins, especially in genetic differentiation of p26.

Corresponding Author: Kawser Ahmed, Department of Fisheries, University of Dhaka, Dhaka-1000, Bangladesh.
Tel: +880-2-9661920 ext. 7779, E-mail: kawser@univdhaka.edu.

Adult *Artemia* lack several components of stress resistance that are of major importance in encysted embryos, namely the small heat shock protein, p26. Lack of p26 in larvae, immature and adults and failure to induce its synthesis in these stages, in spite of concerted efforts, seems strange since p26 is an excellent molecular chaperone. Abundant p26 may not be compatible with cells undergoing division, since the absence of DNA synthesis and cell division are unique features of encysted embryos compared to all other life cycle stages. If the preceding happens in *Artemia*, the genes responsible for the synthesis of p26 which are active during the formation of the encysted gastrula embryo must be kept silent in all other stages. So, we will study the genetic variability of small heat shock protein 26 in different adult *artemia* species. The study will analyze the nuclear DNA variation of p26 in adult *Artemia* species.

Genetical Characterization of *Artemia*

Genetic Approach to Evolution: *Artemia* is exceptionally well studied for the evolutionary processes such as different adaptations, marked biogeographic patterns, speciation, genetic or morphometric differentiation and the interaction between asexuality and parthenogenesis [6, 7]. Many of the factors thought to be responsible for genetic differentiation and speciation in other organisms [8] are observed in *Artemia* viz. ecological isolation, formation of clines (in the content of heterochromatin), polyploidy, heteroploidy and aneuploidy, parthenogenesis [9, 10] and pre-mating or post-mating reproductive isolation [6]. Within their biological communities there is considerable diversification with respect to permanence, seasonality and predictability of the environment [11]. From an evolutionary perspective in particular, different adaptations, distinctive genetic features and marked biogeographic patterns are all found in the genus, thus offering unique opportunities for studies on phylogeny and the interaction between sexuality and parthenogenesis [7]. Morphometric and/or morphological, life history and genetic divergences are widely partitioned both within and between the different reproductive modes [12]. Allopatric divergence and ecological specialization are believed to have shaped *Artemia* evolution, while the influence of dispersal on contemporary regional distributions has only recently been explored [13].

Mutational and ecological models provide more or less specific predictions regarding the genetic and life history architecture of unisexual taxa, the patterns of variation in nuclear and organelle DNA and the timing of consequences due to loss of sexuality [14-16]. In addition, the rate and mode of origin of parthenogenesis may be crucial in determining levels of genetic diversity, the geographic distribution and ecological success of asexual populations [17 -22]. Baxevanis *et al.* [23] has inferred the phylogeny of sexual and parthenogenetic *Artemia* with cosmopolitan distribution, marked geographic and ecological patterns. Newly derivative ITS1 sequences and 16S RFLP markers were jointly analyzed from global isolates indicating significant inter-specific divergence as well as pronounced diversity for parthenogens, matching that of sexual ancestors. Furthermore *Artemia* populations both bisexual and parthenogenetic forms were compared using morphometric and genetic characters [24] and nucleotide divergence and genetic structuring patterns has been clearly distinguished from parthenogenetic populations [25].

Morphometry and Cross Breeding Test: Morphological traits, morphometry analysis and cross breeding test have been described for *Artemia* populations and species studies [26-28]. Many studies show that there are morphological differences among the individuals of different species of *Artemia* [29-34]. Similar differences were reported in morphometry of both sexual and asexual *Artemia* populations from different parts of the world when reared under different environmental conditions [35, 36]. Significantly differences were found of *A. farnciscana* populations from each other at three localities in Argentina on the basis of the measurement of cyst diameter and nauplii length [37, 38]. Moreover biometry of *A. franciscana* were studied mainly strain specific [39], since adult morphometrical traits were described under influence of environmental factors. It is complicated to select and use the best traits for genetic or environmental determination [23,40].

Karyology: *Artemia* chromosomes are analyzed for *Artemia* taxonomy. The euploid chromosome number in most bisexual *Artemia* species is 2n=42 (i.e. *A franciscana, A. salina, A. sinica, A. tibetiana*) while it has been found to be 44 in *A. persimilis* [41]. The ploidy level in the parthenogenetic *Artemia* populations varies from diploidy (2n=42), triploidy (3n=63), tetraploidy (4n=84) to pentaploidy (5n=105) while heteroploidy and aneuploidy are very common phenomena at least in nauplii [9, 42 - 46]. Chromosome analysis in *Artemia* species is a rather difficult task as chromosome spreads are not easily obtainable, especially in polyploidy cells and this becomes more difficult when chromosomes are interconnected by filamentous heterochromatic bridges

(provided that these formations are not artefects). Moreover, nearly all chromosomes lack centric constrictions (diffused centromeres) and the homologous chromatids are not usually conspicuous due to supercoiling or spiralization [45, 47, 48]. Because of these problems, *Artemia* karyotypes or karyograms have been described only very occasionally [45, 46, 49, 50].

Chromocentres are heavily stained heterochromatic areas with highly repetitive DNA family of the type Alu I (110bp), also named satellite I, in the interface nuclei I [51]. This genome trait varies between and within species and is correlated to some extent with genetic differentiation based on Nei's distances [52, 53] as well as with the amount of repetitive DNA. For example, *A. franciscana* shows the highest concentration of repetitive DNA and also shows the highest chromocentre frequency. No such structures have been reported in old world species such *A. salina* and *A. urmiana*, nor in parthenogenetic populations. New world species, in particularly *A. franciscana,* would then represent a derived state likely to have arisen by sequence amplification from an original common ancestor by a mechanism yet not fully understood (unequal crossing over according to Badaracco *et al.* [54]. Differences in chromocentre numbers and staining pattern in *A. franciscana* would be an indication of chromosome reorganizations, either through heterochromatin modification or differential amounts of repetitive DNA, or both of these. This change does not seem to imply reduced hybrid viability or sterility [55] as the reported existence of hybrids (with *A. persimilis*) might suggest. Moreover, the observed correlation between chromocentre frequency and latitude [56] suggested a causative involvement in speciation.

Allozyme Study: The first phylogenetic study on the genus *Artemia* were discussed mainly on the basis of allozymes studies of strains and species [6, 9, 52, 54, 57]. Spatial and temporal changes in allele frequencies have been monitored with allozyme studies for identify the intraspecific population structure and inter-species differences of *Artemia*, as well as to evaluate the importance and relative magnitude of the various evolutionary forces behind.

The two major geographical separations (old versus new world species) were shown the greatest differentiation (D=1.497-1.952) due to Nei's genetic distance [9]. Similar amount of divergence (D=1.073) was observed between *A. franciscana* and *A. persimilis*. Genetic distance value was ranged from 0.254 to 0.808 in favor of the inter-specific comparisons within the old world populations [31, 58].

Recently studies of allozyme electrophoresis techniques allows to compare a handful of randomly selected loci and hence a small portion of the genome (protein-coding loci), whilst a significant part (most in non-coding regions) is not acquiescent to analysis. Moreover, allozyme loci are expected to be loosely linked to Quantitative Trait Loci (QTL) or gene groups directly related with speciation [59, 60].

Molecular Characterization of *Artemia:* Variety of *Artemia* strains and cysts are reaching the world market due to economic profitability in the aquaculture sectors [28, 61]. It is necessary to develop the overall quality of a cyst sample, nauplii and adults because of the rising demand in hatchery [62]. Recently, different popular molecular tools are using for the analysis of the population genetics, phylogeographical and evolutionary studies on *Artemia* [3]. Molecular characterization of *Artemia* have been studied based on random amplified polymorphic DNAs (RAPD), restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphism (AFLP), Denaturing Gradient Gel Electrophoresis (DGGE) and mitochondrial or Nuclear DNA sequences such as ITS-1, HSP26, COI, 12S and 16S mtDNA [3, 23, 27, 34, 63, 69]. First microsatellite DNA markers for the genus *Artemia* have been isolated by Muñoz *et al.*[68]. Hatching cysts, rearing larvae to adulthood samples are used for the molecular characterization of *Artemia*, which is followed by the characterization of adult specimens through crossbreeding test, morphometric study and/or molecular markers analysis [3].

DNA Techniques: Nowadays, several modern technique have brought into play new molecular markers, i.e. RFLPs, RAPDs, AFLPs etc. conveying greater sequence of information than allozymes and their utility has boosted our knowledge of the evolutionary mechanisms operating at the different levels of hierarchy [57, 63, 64, 70, 71]. Denaturing Gradient Gel Electrophoresis is one of the important methods which is used for gene analysis of small crustaceans i.e. *Brachionus, Artemia* etc. [3, 72, 73]. Different molecular methods are using for species identification and/or genetic characterization in *Artemia*.

Restriction Fragment Length Polymorphism-RFLP: It is one of the molecular techniques that DNA is cut with restriction enzymes and amplified through PCR method, electrophoresed (agarose gels), blotted to membranes and probed with cloned radiolabelled DNA that binds to a

single locus. Alleles differing in the presence or absence of nearby restriction sites will produce different fragment sizes. This technique has shown its advantage in authentication of cyst sample [66], but its reliability depend on mixed samples remains uncertain unless individual cysts are analyzed, which is labour-intensive and costly. RFLP technique has high discriminating power, it is reproducible and simple to perform, but expensive and time consuming [74], while, Beristain *et al.* [75] observed the species specific RFLP pattern in *Atemia* HSP26 gene and mentioned that this pattern is a simple and cost-effective single locus tool for genetic analysis.

Amplified Fragment Length Polymorphisms (AFLP): Amplified fragment length polymorphism DNA fingerprinting is a firmly established molecular marker technique, with broad applications in population genetics, phylogenetics, linkage mapping, parentage analysis and single-locus PCR marker development. Technical advances have presented new opportunities for data analysis and recent studies have addressed specific areas of the AFLP technique, including comparison to other genotyping methods, assessment of errors, homoplasy, phylogenetic signal and appropriate analysis techniques [76]. However, AFLP-PCR is s highly sensitive method for detecting polymorphisms, which is previously described [64, 77, 78] used this marker for genetic study of *Artemia* species and found a higher number of loci and polymorphism was significantly higher. Howver, it is time consuming and expensive.

Randomly Amplified Polymorphic DNA (RAPD): Randomly amplification polymorphic DNA technique is a type of PCR reaction, but the segments of DNA amplifies randomly. An arbitrary oligonucleotide of about 10 bases used in PCR reaction will usually anneal well enough to serve as both forward and reverse primer at 3-10 sites. The products are separated and visualized by electrophoresis through agarose and stained. Several scientists and researchers has been used the RAPD techniques to investigate genetic variations among *Artemia* species [6, 27, 63, 65, 71, 79]. Genetic distance value of *A. franciscana* from *A. salina, A. sinica and A. persimilis* are 1.061, 1,105 and 1,165, respectively through RAPDs method by Badaracco *et al.*[63]. *A. persimilis* is more distant from *A. salina* (1.287) and *A. sinica* [80] (1.257), whilst *A. salina* and *A. sinica* are separated by a distance of 1.173. RAPD is a rapid, precise and sensitive method of detection of nucleotide variation to use for good taxonomic investigations at populations, species and genus level. However, Zhanjiang [74] reported reproducibility problems because bands present in one individual may not be present in others.

Denaturing Gradient Gel Electrophoresis-DGGE: Denaturing gradient gel electrophoresis (DGGE) has been shown to detect differences in the melting behavior of small DNA (or RNA) fragments (200-700bp) that differ by as little as a single base substitution. Researchers have found that certain denaturing gels are capable of inducing DNA to melt at various stages, resulting the DNA spreads through the gel and can be analyzed for single components, even those as small as 200-700 base pairs. By comparing the melting behavior of the polymorphic DNA fragments side-by-side on denaturing gradient gels, it is possible to detect fragments that have mutations in the first melting domain. Placing two samples side-by-side on the gel and allowing them to denature together, researchers can easily see even the smallest differences in two samples or fragments of DNA. Differing sequences of DNA will denature at different denaturant concentrations resulting in a pattern of bands separated qualitatively according to their base sequence difference. Once generated, fingerprints can be uploaded into databases in which fingerprint similarity can be used to investigate intra-specific variation within populations [73, 81].

Successfully PCR-DGGE technique has been applied for the study of the phylogenetic utility of the 16S rRNA fragment in elucidating evolutionary relationships within the *Brachionus plicatilis* species [82]. More recently, PCR-DGGE technique was used as a tool for *Brachionus* sp. strain characterization on the basis of nucleotide sequence variation within the 16s rRNA gene [72, 73].

Van Stappen [3] firstly investigated the PCR-DGGE technique for the genetic biodiversity analysis of different *Artemia* species. He conducted of those experiments on the feasibility of this method for quantitative assessment of the respective components of the cyst mixtures consisting of *A. fransiscana* and *A. sinica* in different proportions. He showed a typical band pattern in the DGGE gel for the species which was using the different DNA dilution, but undiluted DNA sample resulted in several weak and blurry additional bands having no clear link with one of the species analyzed. Van Stappen [3] also found that the PCR-DGGE technique was consistent in detecting the presence of *A. franciscana* and pathenogenetic *Artemia* in the samples. However, it was considered to be semi-quantitative at most, with detection limit of about 5% and inconstancy of band intensity on

the gel. Finally he recommended to further methodological improvement in order to overall discriminatory power and reliability.

Mitochondrial and Nuclear DNA: The *Artemia* genome has been mainly studied in *A. franciscana* from San Francisco Bay [83]. With regard to extra-nuclear DNA, the complete mitochondrial DNA of *A. francicana* has been sequenced. Many molecular approaches, analysis of ribosomal DNA and mitochondrial DNA are among the simplest study. Ribosomal DNA sequences (16S rDNA) have been used to establish the phylogenetic relationship in Crustacea including *Artemia* [84] and mitochondrial DNA sequences [Cytochrome oxidase I (COI) and cytochrome b] have also been used to determine bisexual and parthenogenetic *Artemia* strains [57]. Mitochondrial DNA is also used to detect the relationship between *A. franciscana* and *A. persimilis* [67]. Moreover, Lin Hou [85] used nuclear DNA of internal transcribed spacer 1 (ITS 1), located between 18S and 5.8S genes and mitochondrial COI gene as a molecular markers. He used those markers to estimate the genetic relatedness of bisexual *Artemia* populations living in different regions of the world to provide more detail information on the origin of *A. sinica* and to determine the taxonomic status of the two bisexual *Artemia* strains from Tibet and Kazakhstan. Furthermore, mtDNA and nuclear DNA for the molecular phylogenetics and asexuality in the brine shrimp *Artemia* of different species were srudied [23]. Mitochondrial DNA marker was observed in the genetic variation of *Artemia parthenogenetica,* while the complete mitochondrial DNA sequence is available from other *Artemia* species [86]. Distinguished nucleotide divergence and genetic structuring patterns, suggest that may be genetic differentiation existed in *A. parthenogenetica* populations from Iran [25]. He also stated that the genetic composition across different geographical areas would be an important contribution to conservation policies and population management. This mitochondrial DNA segment has been used previously to authenticate commercial cyst sample of brine shrimp [66] and to identification of genetic differentiation of *A. urmiana* from various populations of Urmia Lake [87].

The bisexual *A. urmiana* exhibited a 100% separation from the parthenogenetic populations. However, a 1500 bp mitochondrial DNA fragment showed RFLP patterns for all Iranian populations confirming earlier reports of a close genetic relationship between *A. urmiana* and parthenogenetic *Artemia* [24]. The result of the bisexual and parthenogenetic *Artemia* from Iran are in agreement with the findings of Bossier *et al.*[66]. This finding also revealed that the mtDNA (based on RFLP analysis) does not change during animal development; so it is not a problem if adults or encysted embryos are analyzed. Also, it could be assumed that neutral genetic markers are unaffected by the rearing conditions, thus providing a reliable approach in distinguishing different populations. Moreover, Very recent studies [23] based on joint analysis of ITS1 sequences and 16S rRNA RFLP markers from global isolates of *Artemia* population indicated significant interspecific divergence as well as pronounced diversity for parthenogens, matching that of their sexual ancestors. According to the same study, on the basis of nuclear DNA sequences and cytoplasmic markers, a genetically diverse assemblage of parthenogens in inferred in close affinity with Asian bisexual *Artemia* (*A. sinica, A. urmiana* and *A. tibetiana*). The same study demonstrated the presence of a common 16S rRNA haplotype, indicating that a number of clones may have captured mtDNA from *A. urmiana* or *A. tibetiana.*

Characterization and Functional Role of Heat Shock Protein 26 in *Artemia*

Heat Shock Proteins (HSPs): Heat shock genes are a subset of a larger group of genes coding for molecular chaperones, i.e. proteins that are involved in "house-keeping" function in the cell. Apart from this function, molecular chaperones are involved in transport, folding, unfolding, assembly and disassembly for multi-structured units and degradation of misfolded or aggregated proteins [88]. These tasks are important under normal cellular conditions, however, the need for molecular chaperones is accelerated under useful conditions that could potentially damage the cellular and molecular structures in the cells. Sørensen *et al.* [88] reported that stress resistance including mild stress, effects of high larval densities, inbreeding and age on HSP expression, as well as on natural variation in the expression of HSPs. Analysis revealed that heat shock genes and their products can play an important role in the physiology, ecology and evolutionary process of populations.

Heat shock proteins (HSPs) are induced when a cell undergoes various types of environmental stresses like heat, cold and oxygen deprivation etc. The heat shock response is induced by a range of stressful conditions, such as: (1) environmental stressor-high temperature, low temperature, UV radiation, heavy metals, pesticides, hypoxia, salinity, high density bacterial and viral infection,

parasitism, physical activity, desiccation and oxidative stress etc., (2) genetic stressor-senescence, inbreeding and deleterious mutations [89, 90]. However, the best empirical evidence of HSPs was recorded due to its effects on heat stress resistance. Several families of HSPs have been identified and named according to their molecular weight in kDa. The families consist of one to several closely related genes. Major families are HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (so-called sHSPs of sizes below 30 kDa) and smaller co-factors.

***Artemia* Heat Shock Response:** *Artemia* display heat shock responses and synthesize proteins typical of other organisms experiencing thermal stress. In pioneering work [91, 92], it was demonstrated that *Artemia* cysts are more thermotolerant than newly hatched nauplii. Heat shock delayed cyst development in proportion of stress severity and was influenced by the time the insult was administered. Sub-lethal heat shock generated thermotolerance in Artemia larvae, reaching maximum levels in 4 h, but enhanced heat resistance beyond the high level displayed by cyst was not induced [93]. The synthesis of most larval proteins decline upon exposure to elevated temperature, although proteins of 89 and 68 kDa increase [91, 92]. In contrast, sub-lethally heated *Artemia* cysts maintain synthesis of all proteins to some extent as do cells experiencing induced thermotolerance, signifying they are naturally preconditioned to withstand stress. Enhanced heat tolerance was explained by the presence of molecular chaperones in addition to those occasioned by heat, a role ascribed to the 31kDa protein synthesized constitutively in cysts and induced by heat in larvae [91].

The heat shock responses of *A. franciscana* from the San Francisco Bay salterns in California and the Vinh Tien Shrimp-Salt Cooperative in Vietnam have been examined [94]. First generation Vietnamese adult brine shrimp are more tolerant to heat than are San Francisco Bay *Artemia*, but the molecular basis of enhanced resistance, almost completely lost by the second generation, was not apparent upon comparison of cell free extract proteins by electrophoresis and probing of western blots. Thermotolerance persisted longer in animals from Vietnam and was accompanied by production of HSP70, HSP 67 and unidentified proteins. p26 synthesis was not generated by heat in Vietnamese shrimp, as is the situation for *A. franciscana* from Great Salt Lake [95].

The disaccharide trehalose potentially contributes to thermal tolerance [91, 92] and aerobically incubated *Artemia* cysts synthesize trehalose at 42°C [96], although its effect on thermotolerance was not determined. Trehalose may also function independently as a chemical chaperone or modulate the activity of other molecular chaperones. Supporting the latter proposal, trehalose promotes release of pig heart citrate synthesis from p26 in molecular chaperone assays done *in vitro* [97].

P26, a Small Heat Shock Protein in *Artemia*: p26 chaperones tubulin activity was found in *Artemia* [98] which exposes hydrophobic regions as it "ages" [99]. A protective relationship was demonstrated between a small heat shock/α-crystallin protein and tubulin from the same physiologically stressed organism. Although p26 was suppressed denaturation, heated tubulin failed to assemble normally, most likely because the proteins were associated with one another. Accessory factor(s) are required to separate chaperone and substrate trehalose [97] and ATP [100].

The ability of p26 to confer thermotolerance on living organisms was also investigated. Well synchronized, cyst-derived first instar larvae containing p26 are more heat tolerant than first instar larvae developed directly from fertilized oocytes and devoid of p26 [95]. According to Thomas ([101], metabolic activity in diapause cysts is very low and these embryos are remarkably resistant to physiological stresses. Encysting embryos, but not those undergoing uninterrupted development, synthesize large amounts of two proteins, namely p26 and artemin. Cloning and sequencing demonstrated p26 is a small heat shock/α-crystallin protein, while artemin has structural similarity to ferritin. HSP26 exhibits molecular chaperone activity *in vitro*, moves reversibly into nuclei during stress and confers thermotolerance on transformed organisms, suggesting critical roles in cyst development. Encysted *Artemia* also contain an abundance of trehalose, a disaccharide capable of protecting embryos. *Artemia* represent a novel experimental system where the developmental functions of small heat shock/α-crystallin proteins and other stress response elements can be explored.

Thomas [101] also demonstrated that, *Artemia* has revealed the developmentally regulated synthesis of p26, a small heat shock/α-crystallin protein that exhibits molecular chaperone activity *in vitro* and confers thermotolerance on other organisms and cells. The protein

is thought to play critical roles in embryo encystment, diapause and quiescence, in agreement with the proposal that heat shock gene up-regulation is common to dormancies of different kinds [102]. Genes are required during *Artemia* embryo encystment, diapause and quiescence whereas p26 gene must be regulated. In other organisms, genes are silenced, others up-regulated and some not affected during diapause [102]. This is undoubtedly true for *Artemia* and due to the depth of diapause and quiescence in this organism, all genes may be turned off, an issue yet to be resolved.

Molecular structure of HSP26 in *Artemia:* HSP26 is a small heat shock/α-crystallin protein composed of polypeptide subunits of 26kDa [103] and native p26 exists as oligomers with a molecular mass of approximately 700 kDa [104]. HSP26 was purified to apparent homogeneity as a prerequisite for molecular characterization and assessment of its role in protection of *Artemia* embryos [104]. Partial p26 sequencing by Edman degraded after digestion and fragment recovery by HPLC revealed a small heat shock/α-crystallin protein. This finding was verified by p26 cDNA sequencing and additional Edman degradation with the entire 192 amino acids residues, except for the initiator methionine was determined by both methods [104].

HSP26, the first crustacean small heat shock/ α-crystallin protein was sequenced and the characteristics of this small protein were possesses a conserved α-crystallin domain flanked by variable regions [105, 106]. The amino terminal region of p26 has 10 glycines housed in a peptide of 22 residues within the first 29 amino acids and another starch of 10 residues with 6 arginines, both unique properties within the small heat shock/α-crystallin protein family. Equally interesting to these sequence peculiarities were identified, because p26 undergoes nuclear translocation, is the absence of a typical nuclear localization signal. The predicted secondary structure of p26 which has a calculated monomer molecular mass of 20.7kDa, is predominately â-sheet. The protein exhibits quaternary structure forming oligomers as large as 700kDa and containing 34 subunits [104]. Glycines and arginines in the p26 sequence constitute novel characteristics of the small heat shock/α-crystallin protein.

Function of HSP26: Heat shock protein p26 encode the cytosolic members of the small heat shock protein (sHSP) family of molecular chaperones ([107]. sHSPs bind and prevent unfolded substrate proteins from irreversibly forming large protein aggregates. Bound substrate proteins can eventually be released and refolded in either a spontaneous or chaperone-assisted manner [108]. HSP26 activity is found only under stress conditions, this target substrate profiles of this chaperones overlap by approximately 90% [109]. Null mutation in HSP26 cause abnormal cell morphology that resembles the effects of dehydration, aging, cytoskeleton damage or cell wall damage [109].

The heat shock protein 26 transcript is undetectable in unstressed cells, but is strongly induced by heat shock, salt shock, cell cycle arrest, nitrogen starvation, carbon starvation, oxidative stress and low pH [108, 110, 111]. Amoros and Estruch [111] reported that under these conditions of stress, HSP26 expression is upregulated by the transcription factors Hsf1P and Msn2p/Msn4p which, respectively bind heat shock elements and stress elements found in the HSP26 promoter.

12 HSP26 dimers assemble to form a 24 sub-unit homo-oligomeric complex shaped like a hollow sphere [112, 113]. Elevated temperature is required to active this complex that means heat shock causes a conformational change in HSP26 that enables the complex to bind substrate proteins [114]. Higher temperatures also coincide with the disassociation of the complex back into dimmers and it had been thought that these dimers were the active form of HSP26. However, recently it has been shown that dimer reformation is not necessary for HSP26 activation and chaperone function [112, 115].

Heat shock proteins are normally found inside cell. When they are found outside the cell, it indicates that a cell has become so sick that it has died and spoiled out all of its contents. This kind of messy, unplanned death is called necrosis and only occurs when something is very wrong with the cell. Extracellular HSPs are one of the most powerful ways of sending a 'danger signal' to the immune system in order to generate a response that can help to get rid of an infection or disease.

During *Artemia* development, a small heat shock/α-crystallin protein-HSP26 is synthesized within the five days from oocyte fertilization to release of encysted embryos and it is occurred only oviparous pathway [93, 95, 103, 104, 116, 117, 118, 119]. Before release of cysts from females, embryo exhibit HSP26 mRNA on the second day following fertilization and the massage peaks at day four post-fertilization [95]. HSP26 initially appears on day three after fertilization and is present in near maximum

amounts at the time of cyst release [95, 117]. Embryos undergoing ovoviviparous development, however, synthesize neither p26 mRNA nor contain the protein. Both nuclei and cytoplasm of oviparous *Artemia* embryos possess p26, which begins to move into nuclei three days post fertilization, a process that persists after cyst release [95, 117]. Although p26 has not been precisely localized in nuclei of diapause embryos, sometimes it is found in discrete intranuclear compartments or foci of activated cysts [118]. Moreover, p26 is reversibly translocated into nuclei under stress conditions such as anoxia, heat shock and diapauses, perhaps in response to reduced intracellular pH [93, 103, 116]. Nuclear localization suggests that p26 affects transcription, thereby contributing to metabolic inhibition as cysts enter diapauses. Another possibility is that p26 prevents DNA replication, mitosis and cytokinesis, none of which occur during encysted growth, although all resume upon emergence [120, 121]. Disappearance of p26 mRNA is well underway five hours after activated cysts resume development and the massage is all, but gone when nauplii emerge [95]. HSP26 is detectable on western blots of cell-free extracts from *Artemia* up to instar-I larvae, but not in extract from instar-II [95, 103]. Immunoflurescent staining reveals a progressive loss of p26 from *Artemia* larvae, with the protein last detected in a subset of salt gland nuclei [95]. The significance of this latter observation is not clear, but the salt gland cells containing p26 do not divide. The salt gland undergoes developmentally programmed disappearance and is missing from adult brine shrimp. HSP26 may contribute to salt gland loss, perhaps by modulating transcription and/or DNA replication, or it may linger because these cells are facing apoptotic stress. However, the presence of small heat shock/α-crystallin in cells indicates that differentiation and growth of the cell rather not the programmed cell death i.e., apoptosis [122, 123, 124].

In conclusion, the survival of *Artemia* is affected by different factors such as salinity, temperature, ionic composition and biotic interaction, etc. It is evident that heat shock genes and their products can play an important role in the physiology, ecology and evolutionary process of *Artemia* populations. The heat shock protein 26 of *Artemia* is one of the important heat shock protein which is directly involved to development of cyst, diapauses, quiescence and embryo and to survive larval and adult stage in different harsh environment.

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