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MINI REVIEW

Stress tolerance during diapause and quiescence of the brine shrimp, *Artemia*

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Abstract Oviparously developing embryos of the brine shrimp, *Artemia*, arrest at gastrulation and are released from females as cysts before entering diapause, a state of dormancy and stress tolerance. Diapause is terminated by an external signal, and growth resumes if conditions are permissible. However, if circumstances are unfavorable, cysts enter quiescence, a dormant stage that continues as long as adverse conditions persist. *Artemia* embryos in diapause and quiescence are remarkably resistant to environmental and physiological stressors, withstanding desiccation, cold, heat, oxidation, ultraviolet radiation, and years of anoxia at ambient temperature when fully hydrated. Cysts have adapted to stress in several ways; they are surrounded by a rigid cell wall impermeable to most chemical compounds and which functions as a shield against ultraviolet radiation. *Artemia* cysts contain large amounts of trehalose, a non-reducing sugar thought to preserve membranes and proteins during desiccation by replacing water molecules and/or contributing to vitrification. Late embryogenesis abundant proteins similar to those in seeds and other anhydrobiotic organisms are found in cysts, and they safeguard cell organelles and proteins during desiccation. *Artemia* cysts contain abundant amounts of p26, a small heat shock protein, and artemin, a ferritin homologue, both ATP-independent molecular chaperones important in stress tolerance. The evidence provided in this review supports the conclusion that it is the interplay of these protective elements that make *Artemia* one of the most stress tolerant of all metazoan organisms.

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Introduction

Embryos of the crustacean, *Artemia*, undergo either ovoviviparous or oviparous development, respectively, yielding swimming larvae (nauplii) or gastrula stage embryos enclosed in a rigid chitinous shell (cysts) (Liang and MacRae 1999; MacRae 2003; Dai et al. 2011a; Ma et al. 2013). Upon liberation from females, cysts undergo profound reduction in metabolism and enter diapause, a state of developmental arrest and greatly enhanced stress tolerance (Clegg 1967, 1997; Clegg and Jackson 1998; King and MacRae 2012; Duan et al. 2014). Diapause occurs in animals other than *Artemia*, most commonly in insects, and initiates in anticipation of adverse environmental conditions signaled by day length, perhaps via changes in circadian clock gene expression (Nambu et al. 2004; MacRae 2010; King and MacRae 2015; Meuti et al. 2015).

Entering diapause promotes organismal survival during exposure to temperature fluctuation, desiccation, and hypoxia/anoxia. Animals remain in diapause, even in conditions favorable for growth, until a habitat-specific, exogenous signal like desiccation, cold, elevated CO_2, or light terminates the dormancy, after which growth resumes (Robbins et al. 2010). The need for a termination cue differentiates diapause from quiescence, a hypometabolic, stress-tolerant state requiring only return to favorable growth parameters for termination (Drinkwater and Clegg 1991). Stress tolerance during diapause and quiescence in *Artemia* has been examined from several perspectives including the synthesis, composition, and structure of the cyst shell (Liu et al. 2009; Dai et al.

2011a; Ma et al. 2013), the role of trehalose (Clegg 1965; Clegg and Jackson 1992; Yang et al. 2013), the composition and activity of late embryogenesis abundant (LEA) proteins (Sharon et al. 2009; Wu et al. 2011; Toxopeus et al. 2014), and the function of molecular chaperones such as small heat shock proteins (sHsps) and artemin (King and MacRae 2012; King et al. 2013, 2014).

The stress tolerance of diapause and quiescent *Artemia* cysts, dependent on a variety of interacting adaptations, surpasses that of most, if not all other metazoans. The examination of stress-related adaptations functioning either in isolation or in concert contributes to our understanding of diapause, quiescence, and other types of dormancies, and for this reason, the information that follows has significance beyond the realm of *Artemia*. Thus, the purpose of this article is to consider the cell/molecular/structural adaptations to stress that enable the survival of *Artemia* cysts during diapause and quiescence (Fig. 1) and to examine these characteristics in the context of other animals that successfully endure exposure to stressors that would normally be expected to end their lives.

Structure and function of the *Artemia* cyst shell

The shell of *Artemia* cysts originates from three pairs of shell glands formed by linked pairs of oblong cells that interact with the uterus via a cellular duct composed of a neck cell and usually three duct cells (Anderson et al. 1970; Criel 1980; Criel and MacRae 2002). Variation in the terminology identifying the layers of the cyst shell may reflect true ultrastructural distinctions or different preparative techniques, and the shell is modified during post-diapause development further complicating the interpretation of structural data (Rosowski et al. 1997; Sugumar and Munuswamy 2006; Dai et al. 2011a). The layers of the shell are, from the exterior of the cyst to the interior, the cortical and alveolar layers of maternal origin which shape the non-cellular chorion, and the outer cuticular membrane, the chitinous fibrous layer, and the inner cuticular membrane of embryonic origin, establishing the embryonic cuticle (Fig. 2) (Morris and Afzelius 1967; Hofmann and Hand 1990).

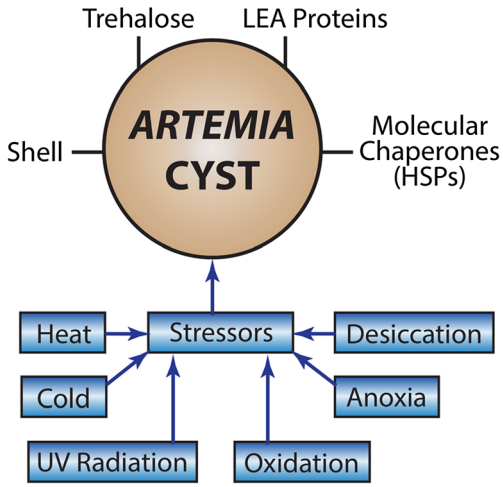

Fig. 1 Adaptations to stress in *Artemia* cysts. When in diapause and quiescence, *Artemia* cysts may experience stressors (*blue boxes*) and several cyst-specific characteristics/mechanisms have evolved to offset these adverse environmental and physiological conditions. *Artemia* cysts are surrounded by a rigid, semi-permeable shell, a morphological adaptation that resists UV radiation and is the first line of defense for the enclosed embryos. The disaccharide trehalose is synthesized by diapause-destined embryos and, with LEA proteins, is thought to be especially effective in protecting cysts against desiccation. The molecular chaperones p26 and artemin prevent irreversible protein denaturation during stress, acting as a first-line, molecular-level defense. These adaptations function in concert, endowing *Artemia* cysts with a degree of stress tolerance seldom seen in any life history stage of any other animal

Removal of the outer shell layer by decapsulation correlates with increased sensitivity to ultraviolet (UV) light, faster water loss, and reduced tolerance to drying and rehydration (Morris and Afzelius 1967; Anderson et al. 1970; Tanguay et al. 2004; Clegg 2005; Liu et al. 2009; Dai et al. 2011a; Ma et al. 2013). The outer cuticular membrane is impermeable to most nonvolatile compounds including amino acids, glycerol, glucose, inorganic phosphate, and uracil, although H_2O and CO_2 penetrate this layer (Clegg 1966, 1967; Golub and Clegg 1968; Clegg and Golub 1969; De Chaffoy et al. 1978). Shell impermeability, acquired in the uterus, is a prerequisite for the normal growth of *Artemia* embryos, implicating the shell gland in development (De Chaffoy et al. 1978).

Subtractive hybridization revealed diapause-related chitin-binding protein messenger RNAs (mRNAs) in embryos of *Artemia franciscana* (Qiu et al. 2007) and in oocytes of *Artemia parthenogenetica* (Dai et al. 2011b), but their translation was not studied. Complementary DNAs (cDNAs) encoding upregulated chitin-binding proteins, Arp-CBP-A,

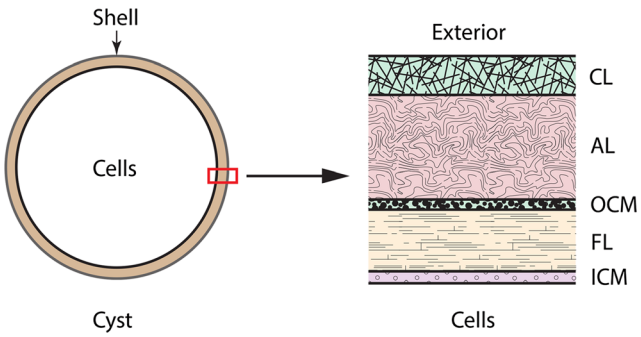

Fig. 2 Schematic representation of the *Artemia* cyst shell. A cyst in cross section is shown in the *left side of the diagram*. The *right side of the figure*, showing the ultrastructure of the cyst shell, is an enlargement of the portion of the shell enclosed in the *red box*. *CL* cuticular layer, *AL* alveolar layer, *OCM* outer cuticular membrane, *FL* fibrous layer, *ICM* inner cuticular layer. The relative widths of the cyst shell layers shown in the figure approximate the relative widths of the layers seen in samples prepared for electron microscopy. The figure was adapted from Drinkwater and Clegg (1991)

Arp-CBP-B, and Arp-CBP-C, were subsequently cloned from oviparous oocytes of *A. parthenogenetica* (Ma et al. 2013). mRNAs specifying chitin-binding proteins are produced in diapause-destined embryos prior to cyst release from ovisacs, with *Arp-CBP-A* mRNA most prevalent and *Arp-CBP-C* mRNA the least common. Ovoviviparous embryos, on the other hand, lack significant amounts of the chitin-binding protein mRNAs. The Arp-CBPs are components of the embryonic cuticular layer with Arp-CBP-A in the inner cuticular and fibrous layers and Arp-CBP-B and Arp-CBP-C in the inner and outer cuticular membranes. Individual knockdown of Arp-CBPs by RNAi disrupts the embryonic cuticular layer with the loss of each Arp-CRP affecting outer cuticular membrane structure but not cyst morphology, hatching, and stress tolerance. Simultaneously injecting double-stranded RNA (dsRNAs) for the three Arp-CBPs disturbs all components of the embryonic cuticular layer, demonstrating roles in wall formation and function (Ma et al. 2013).

The chorion, comprised of the maternally derived cortical and alveolar layers, surrounds the embryonic cuticular layer, protecting cysts against environmental stress (Morris and Afzelius 1967; Anderson et al. 1970; Tanguay et al. 2004; Liu et al. 2009; Dai et al. 2011a; Ma et al. 2013). cDNAs representing shell gland specifically expressed genes (*SGEGs*), termed *SGEG1* and *SGEG2*, were isolated from *A. parthenogenetica* by subtractive hybridization (Liu et al. 2009; Dai et al. 2011a). *SGEG1* encodes a single protein with a putative signal sequence, and elimination of SGEG1 by RNAi yields cysts with translucent, soft shells, a deformed chorion layer of half normal thickness, and diminished stress tolerance, signifying a role for SGEG1 in shell structure and function (Liu et al. 2009). About 20 % of cysts lacking SGEG1 hatch spontaneously in the absence of a diapause termination signal. Significant amounts of SGEG1 mRNA reside in the ovisacs of females producing diapause-destined embryos, but only minor amounts appear in ovisacs containing nauplius-destined embryos (Liu et al. 2009). *SGEG1* is expressed exclusively in the shell gland and loss of SGEG1 affects the chorion layer, agreeing with the earlier conclusion that the chorion arises from shell gland secretions (Morris and Afzelius 1967). *SGEG2*, also expressed only in shell gland secreting cells, is cleaved into two proteins, SGEG2a and SGEG2b, found predominately in the cortical and alveolar layers of cyst shells, but not in the embryonic cuticle or embryo cells (Dai et al. 2011a). Knockdown of RNA encoding SGEG1 and SGEG2 yields abnormal cyst phenotypes with those missing SGEG2 having hard shells as opposed to the soft shells of cysts lacking SGEG1.

The cyst shell constitutes a formidable, semi-permeable barrier that protects cysts year round in hostile changing environments. In favor of this idea, cysts lacking SGEGs and with malformed shells are much more susceptible to desiccation, temperature change, and UV radiation than are cysts with intact shells.

Trehalose and stress tolerance in *Artemia* cysts

Water is essential to cell life by way of dynamic interactions with proteins, lipids, and nucleic acids, but *Artemia* cysts survive desiccation and the loss of free water required for metabolism (Clegg 1967, 1974, 1977, 1978). Cysts are ametabolic when hydration is less than 30 g H_2O/100 g cysts. At 35±5 g H_2O/100 g cysts, about 25 % maximum hydration, limited intermediary metabolism involving Krebs cycle intermediates, amino acids, and pyrimidine nucleotides transpires (Clegg 1974, 1976a; Clegg and Cavagnaro 1976). The value of 0.35 g/g is midrange for bound water which is associated with macromolecules and structural surfaces and possesses properties different from bulk water. At 63±6 g H_2O/100 g cysts, metabolism characteristic of fully hydrated cysts, including trehalose degradation, aerobic energy production, and macromolecular synthesis begins, as does development. The rapid initiation of metabolic activity that occurs upon hydration indicates that dormant encysted *Artemia* embryos are primed, and only water, air, and adequate temperature are required for growth when diapause and quiescence terminate (Clegg 1976a, b, c, 1977; Clegg and Cavagnaro 1976).

Artemia cysts endure repeated dehydration/rehydration with little mortality and no adverse effects on transcription, but this treatment kills prenauplii and nauplii (Morris 1971; De Chaffoy and Kondo 1976). Cysts dehydrated to terminate diapause and then rehydrated were examined by electron microscopy, revealing structures common to eukaryotic cells but in a more crowded setting (Morris 1968; Clegg and Trotman 2002). There is little evidence to suggest that cysts accommodate desiccation by changing internal structure, a conclusion reinforced by the similarity in ultrastructure of anoxic and normoxic cysts (Hofmann and Hand 1990; Clegg et al. 2000). Adaptation to desiccation must reside in other cyst elements, with the cyst shell already mentioned as one candidate and trehalose another.

Trehalose (α-D-glucopyranosyl-1-1-α-D-glucopyranoside), a non-reducing disaccharide of glucose, represents about 15 % of cyst dry weight but is absent from embryos developing directly into nauplii (Clegg 1962, 1965). Trehalose production occurs in embryos and not maternal tissues, and the sugar accumulates in cysts even after release from females. Trehalose, glycogen, and, to a lesser extent, glycerol, are plentiful in desiccated cysts and remain remarkably constant upon years of storage, indicating they are metabolically inert; nonetheless, the viability of stored cysts declines (Clegg 1962, 1965, 1967). Accordingly, the inability of stored cysts to hatch, perhaps reflecting death, is due to factor(s) other than the loss of carbohydrates.

Trehalose is abundant in many, but not all, organisms that survive anhydrobiosis, an ametabolic state incurred by desiccation, oxidation, freezing, and heat, and all survived by

Artemia during diapause and quiescence (Crowe et al. 1992; Singer and Lindquist 1998a, b; Tunnacliffe et al. 2005; Ratnakumar and Tunnacliffe 2006; Hengherr et al. 2008; Erkut et al. 2011, 2013; Morano 2014; Tapia and Koshland 2014). Trehalose preserves membranes by blocking fusion, thus averting leakiness, and by lowering transition temperature (T_m), the latter proposed to come about by different but overlapping processes as follows (Crowe et al. 1987, 1992; Clegg and Trotman 2002; Crowe 2007; Erkut et al. 2011; Hengherr et al. 2011). Trehalose may replace water by forming hydrogen bonds with polar residues of phospholipids and proteins, thereby conserving molecular packing in membranes, the so-called water replacement hypothesis. Trehalose could accumulate at the surfaces of macromolecular structures, or vitrification, the creation of amorphous glasses contributing to the maintenance of cell structure, is possible. Whatever the case, lowering T_m maintains membranes in the liquid crystalline rather than gel phase upon dehydration, and because the dried membranes remain in the liquid crystalline state, phospholipid phase transitions leading to membrane leakiness are prevented (Crowe et al. 1983, 1984, 1987).

Trehalose stabilizes proteins and limits their aggregation in vitro and in heat-shocked yeast (Singer and Lindquist 1998a, b). Stabilization favors protein renaturation by Hsp104 which, with trehalose, is strongly induced in moderately stressed and stationary-phase yeast. Hsp104, an energy-requiring molecular chaperone, promotes short-term dehydration tolerance in yeast (Tapia and Koshland 2014). Conversely, trehalose maintains both short- and long-term protein homeostasis by an energy-independent mechanism effective even when organisms experience metabolic depression, desiccation, and ATP limitation, as occur in *Artemia* cysts during diapause and quiescence (Crowe et al. 1987; Tapia and Koshland 2014). Trehalose protects proteins if the chaperone system is overwhelmed or when there is insufficient energy for ATP-dependent chaperones to operate.

Once *Artemia* cysts are activated and metabolism resumes, trehalose is converted to glycogen and glycerol, rather than undergoing complete oxidization, and trehalose is all but gone by the time nauplii hatch (Clegg 1962, 1964, 1965, 1967; Clegg and Cavagnaro 1976). These results suggest trehalose is a major energy source for cysts, an inference bolstered by the identification in cysts of trehalase, the trehalose-degrading enzyme. Trehalase of approximately 70 kDa was prepared from *Artemia* (Nambu et al. 1997), whereas in earlier work, trehalase is shown to exist as proteins of 235 kDa at pH 6.3 and 110 kDa at pH 8.6 (Hand and Carpenter 1986). The larger trehalase has very low activity, and because the intracellular pH of dormant cysts is acidic, trehalose utilization would be limited until diapause or quiescence terminates and pH rises (Busa and Crowe 1983; Hand and Carpenter 1986).

In *A. parthenogenetica*, trehalase is an 80-kDa enzyme found predominately in cysts. The synthesis of trehalase is regulated transcriptionally by *Artemia* trehalase-associated protein (ArTAP), a diapause-specific protein that interacts with an intron in the trehalase (*tre)* gene (Yang et al. 2013). Knocking down ArTAP augments cyst trehalase, induces apoptosis, and leads to abnormal shells devoid of the chitin-binding proteins, Arp-CBP-A and Arp-CBP-C. ArTAP is likely to regulate the trehalase gene, keeping the enzyme at modest levels and, in turn, controlling the utilization of trehalose as cysts develop.

Trehalose clearly contributes to the stress tolerance of *Artemia* cysts, shielding proteins and membranes from damage, especially during dehydration/rehydration. Moreover, trehalose serves as an energy source to sustain embryo development after diapause and quiescence terminate.

LEA proteins and desiccation tolerance in *Artemia* cysts

LEA proteins, often enriched in repeated motifs, are hydrophilic, mostly unstructured, flexible proteins within the group of intrinsically disordered proteins; however, when drying, many LEA proteins reversibly assume structure stabilized by hydrogen bonding and electrostatic interactions (Goyal et al. 2003; Chakrabortee et al. 2011; Hundertmark et al. 2012; Hatanaka et al. 2013). LEA proteins were first discovered in maturing plant seeds (Dure et al. 1981) and have since been found in desiccation-tolerant cyanobacter (Close and Lammers 1993), bacteria and Archaea (Campos et al. 2013), nematodes (Browne et al. 2002; Gal et al. 2004; Goyal et al. 2005a; Erkut et al. 2013), rotifers (Tunnacliffe et al. 2005), an insect (Kikawada et al. 2006; Hatanaka et al. 2013), and a crustacean (Hand et al. 2007; Sharon et al. 2009). LEA proteins are induced by and mediate the effects of dehydration/rehydration (Gal et al. 2004; Tunnacliffe et al. 2005; Kikawada et al. 2006), osmotic stress (Close and Lammers 1993; Gal et al. 2004; Kikawada et al. 2006), temperature fluctuation (Gal et al. 2004), and freezing-thawing (Hand et al. 2011; Campos et al. 2013; Toxopeus et al. 2014). Protein aggregation is inhibited by LEA proteins, either by chaperoning (Grelet et al. 2005) or more probably by molecular shielding, the formation of electrostatic and/or physical barriers that prevent protein interactions (Goyal et al. 2005b; Chakrabortee et al. 2011; Hatanaka et al. 2013). LEA proteins may stabilize membranes by interaction with phospholipids (Jaspard et al. 2012), and they strengthen vitrified sugar glasses of trehalose or sucrose, raising the transition temperature and sustaining glasses upon exposure to increased heat (Hand et al. 2011).

The *A. franciscana* group 1 LEA proteins, LEA-1a, LEA-1b, and LEA-1c, the first of this group found in an animal, are slightly acidic and very hydrophilic consisting of different numbers of characteristic, tandem, 20 residue repeats similar

to NH_2-GGOTRREQLGEEGYSQMGRK-COOH (Sharon et al. 2009). Group 1 LEA proteins are found in the cytosol and mitochondria of diapause-destined *A. franciscana* embryos about 4 days post-fertilization, but they are absent from nauplii, larvae and adults, declining quickly as post-diapause development proceeds (Warner et al. 2010). The group 1 LEA proteins constitute about 1 % of the protein in cyst post-mitochondrial supernatant and they have the potential to protect proteins from drying-induced aggregation by forming glasses with trehalose, an abundant cyst sugar (Sharon et al. 2009; Warner et al. 2010; Toxopeus et al. 2014). Indeed, the amount of group 1 LEA protein in cysts is at a maximum when trehalose is highest and stress tolerance most profound; in this context, the knockdown of group 1 LEA proteins reduces cyst tolerance to desiccation and freezing (Toxopeus et al. 2014). The group 1 LEA proteins, AfLEA1.1 and AfLEA1.3, also occur in *A. franciscana*. AfLEA1.3 preserves mitochondrial function and improves survival of transgenic *Drosophila melanogaster* Kc167 cells during freeze-thaw, drying, and hyperosmosis. AfLEA1.3 protects against drying when hydrated and unstructured, showing that folding is not a prerequisite for LEA protein activity (Marunde et al. 2013).

Group 3 LEA protein cDNAs from *A. franciscana* encode AfrLEA1 and AfrLEA2, cytosolic, hydrophilic proteins of approximately 39 kDa with repeating motifs respectively of 32 and 14 residues (Hand et al. 2007; Boswell et al. 2014). *AfrLEA1* and *AfrLEA2* mRNAs and their corresponding proteins are prominent in quiescent, diapause, and post-diapause embryos of *Artemia*, but not in larvae. Another group 3 LEA protein, AfrLEA3m, is enriched in α-helices, contains repeated motifs, and has a mitochondrial pre-sequence (Menze et al. 2009; Boswell and Hand 2014). *AfrLEA3m* mRNA is more abundant in diapause-destined embryos than in nauplii, and mitochondria containing trehalose and AfrLEA3m display enhanced freeze tolerance. Transfection with *AfrLEA2* and *AfrLEA3m* cDNA under tetracycline (Tet) control, along with a constitutively expressed trehalose transporter 1 cDNA, yields HepG2 cells with AfrLEA2 in the cytoplasm and AfrLEA3m in the mitochondria, with the latter enriched in trehalose (Li et al. 2012). Either trehalose or AfrLEA2 improves membrane integrity and survival of transfected HepG2 cells upon spin drying, with protection reaching 98 % when they are used together. Unexpectedly, AfrLEA3m acting alone increases transfected cell survival to almost this level, indicating that AfrLEA3m is important to mitochondrial integrity upon water loss (Li et al. 2012), a view strengthened by the presence of more group 3 LEA proteins, AfrLEA3m_47, AfrLEA3m_43, and AfrLEA3m_29, in *A. franciscana*, all with mitochondrial localization signals (Boswell et al. 2014). These LEA proteins cross-react with antibody to AfrLEA3m, but their corresponding cDNAs are sufficiently different that each is liable to be the product of a distinct gene.

Fourteen LEA and LEA-like genes were identified in *A. franciscana*, with *AfrLEA1-5*, *AfrLEA3-5*, *AfrLEA3-like1*, and *AfrLEA3-like2* encoding previously unknown LEA proteins (Wu et al. 2011). The amount of mRNA encoded by each gene varies with some mRNAs enriched in cysts and others either unchanged throughout development or increased in larvae. Hypersaline conditions increase the expression of some of these genes, although insignificantly, and others are unaffected by this stress.

Structural diversity, differential synthesis, and multi-organelle localization indicate LEA proteins in *Artemia* cysts are functionally promiscuous, protecting many cell components by different but overlapping mechanisms. The LEA proteins are particularly well adapted to safeguard against desiccation, a stress encountered commonly by *Artemia* cysts during diapause and quiescence.

Molecular chaperones, diapause, and quiescence in *Artemia*

Molecular chaperones, known as heat shock proteins (HSPs) or stress proteins, are essential to cell life. The ATP-dependent chaperones, Hsp90 (Karagöz and Rüdiger 2015), Hsp70 (Mayer 2013), and Hsp60 (Yébenes et al. 2011), fold nascent proteins and either refold or degrade denatured proteins. The sHSPs, on the other hand, are ATP-independent chaperones that prevent irreversible denaturation of other proteins but are not otherwise actively engaged in their subsequent processing (Haslbeck and Vierling 2015). The ATP-dependent chaperones may sequester partially denatured proteins when ATP is limiting and, with the sHSPs, constitute a protective network during diapause and quiescence (King and MacRae 2015). Stress tolerance in cysts depends on molecular chaperones, a proposal first occasioned by the response of *A. franciscana* to heat (Miller and McLennan 1988a, b).

Artemia cysts contain two abundant, low-molecular-mass proteins (Grosfeld and Littauer 1976; De Herdt et al. 1979), one being p26, a sHSP (Clegg et al. 1994, 1995; Jackson and Clegg 1996; Liang et al. 1997a, b). p26, with a calculated monomeric molecular mass of 20.7 kDa, has an α-crystallin domain flanked by variable amino- and carboxyl-terminal regions, and it assembles into oligomers (Liang et al. 1997a; Crack et al. 2002; Sun et al. 2004, 2006; Sun and MacRae 2005). Only diapause-destined *Artemia* embryos synthesize p26, and expression of the *p26* gene is indifferent to heat in spite of possessing up-stream heat shock elements (Liang and MacRae 1999; Qiu et al. 2006; Qiu and MacRae 2008a). p26 mRNA appears in *A. franciscana* embryos 2 days post-fertilization, followed closely by protein, with mRNA lost rapidly during post-diapause development. As nauplii emerge, p26 wanes and is last observed in

salt gland nuclei of second instar larvae (Liang and MacRae 1999).

The lack of p26 in embryos developing directly into nauplii, its abundance in cysts and its degradation as nauplii emerge from cysts, suggests this sHSP functions during diapause and quiescence. Supporting this viewpoint, first instar nauplii hatched from cysts and containing residual p26 are more heat tolerant than nauplii that develop ovoviviparously and lack p26 (Liang and MacRae 1999). Transformed *Escherichia coli* synthesizing p26 gains heat resistance (Liang and MacRae 1999; Crack et al. 2002; Sun and MacRae 2005; Sun et al. 2006), as do stably transfected, mammalian cells, which also resist apoptosis (Villeneuve et al. 2006; Wu and MacRae 2010). Reversible translocation of p26 into nuclei occurs in cysts exposed to anoxia, heat, and acidic pH, and during entry into diapause (Clegg et al. 1994, 1995, 2000; Liang et al. 1997b; Liang and MacRae 1999). p26, which locates to nuclei in transfected mammalian cells (Sun et al. 2004, 2006), is thought to interact with Hsp70 and nuclear lamins in cyst nuclei, but has little effect on transcription (Willsie and Clegg 2001, 2002). Based on these findings, p26 was thought to act as a molecular chaperone, a conclusion bolstered by the observation that p26 purified from either *A. franciscana* (Liang et al. 1997a; Day et al. 2003) or transformed bacteria (Sun et al. 2004, 2006; Sun and MacRae 2005) prevents the denaturation of heated tubulin and citrate synthase, and insulin when exposed to dithiothreitol. Site-directed mutagenesis established the structural stability of p26, a characteristic that prolongs the life of the protein through diapause (Sun and MacRae 2005; Sun et al. 2006; Wu and MacRae 2010).

The screening of subtractive libraries enriched in cDNAs from oviparously developing *Artemia* embryos yielded, in addition to p26, the sHSPs ArHsp21 and ArHsp22 (Qiu et al. 2007; Qiu and MacRae 2008a, b). ArHsp21 and ArHsp22 contain α-crystallin domains, both oligomerize and they are diapause-specific, peaking in cysts but undetectable in instar II larvae. p26, ArHsp21, and ArHsp22 have equivalent chaperone activities in vitro but p26 and ArHsp22 enter nuclei whereas ArHsp21 is found only in the cytosol. Moreover, ArHsp22 is the lone sHSP induced by heat, but only in adults (Qiu and MacRae 2008a). These variations suggest that the three known *Artemia* sHSPs, although similar in several ways, have different functions during diapause and quiescence, a proposal tested by RNAi. The injection of *A. franciscana* females with p26 double-stranded RNA (dsRNA) eliminates p26 from cysts but has no effect on the other identified sHSPs (King and MacRae 2012; King et al. 2013). p26 knockdown by RNAi reduces cyst tolerance to desiccation, freezing, and heat, confirming the earlier deduction that p26 functions as a molecular chaperone in cysts (King and MacRae 2012). Moreover, the loss of p26 slows embryo development and allows spontaneous diapause termination, indicating roles other than in stress tolerance. The knockdown of ArHsp21 has a marginal effect on cyst stress resistance and none on embryo development (King et al. 2013). The injection of *Artemia* adults with ArHsp22 dsRNA causes death, preventing further analysis of ArHsp22 by RNAi and inferring that ArHsp22 has a critical, potentially stress-related activity in adults that is not characteristic of p26 and ArHsp21 (King et al. 2013). That losing p26 impacts cysts more than the loss of ArHsp21 may come about because p26 is 7 % of cyst soluble protein and ArHsp21 is 1.2 % (King et al. 2013); however, the amount of ArHsp21 is sufficiently high that its loss is expected to lower stress tolerance more than observed.

The second abundant cyst protein termed artemin (Slobin 1980) or the 19S complex (De Herdt et al. 1979, 1981) is similar in amino acid sequence to ferritin, but artemin is enriched in cysteine and possesses an extended carboxyl-terminal tail not found in ferritin (De Graaf et al. 1990; Chen et al. 2003; Rasti et al. 2009). Artemin has a monomeric molecular mass of 27 kDa and, like ferritin, assembles into oligomers of 24 subunits (Chen et al. 2007; Hu et al. 2011). In contrast to ferritin, the central cavity of oligomerized artemin is occupied by the carboxyl-terminus of constituent monomers and fails to bind iron, a finding augmented by electron microscopy and the paucity in artemin of di-iron ferroxidase center residues required for interaction with iron (De Graaf et al. 1990; Chen et al. 2003, 2007).

Artemin appears in the cytosol of cyst-destined, but not nauplius-destined, embryos by 4 days post-fertilization (Grosfeld and Littauer 1976; De Herdt et al. 1979, 1981; Tanguay et al. 2004), and it constitutes about 7 % of the soluble protein in mature cysts (King et al. 2014). Most artemin mRNA is gone from early cyst-derived nauplii (Chen et al. 2003) but when the protein disappears is unknown. Artemin is extremely stable, enduring extended cyst storage, years of anoxia, and heating in vitro at 70 °C which causes binding of non-polyadenylated RNA (Warner et al. 2004; Shahangian et al. 2011). Structural stability and the correspondence between abundance and elevated stress resistance imply that artemin protects *Artemia* cysts, a proposition proven by reduced tolerance to desiccation and freezing after knockdown of artemin (King et al. 2014).

Purified artemin prevents heat-induced denaturation of other proteins in the absence of added ATP, an activity shared by ferritin (Chen et al. 2007; Hu et al. 2011; Shahangian et al. 2011). The heat resistance of transformed bacteria synthesizing artemin is enhanced (Rasti et al. 2009), and stably transfected mammalian cells containing artemin have increased tolerance to thermal and oxidative stress (Chen et al. 2007). That ferritin and artemin are both chaperones suggests that artemin evolved from ferritin to produce an abundant, cyst-specific, molecular chaperone that neither sequesters iron nor disrupts iron metabolism, as would happen if large

Table 1 Cyst elements responsible for stress tolerance during diapause and quiescence

Protective element	Characteristics/functions
Shell	Rigid; selective barrier; Arp-CBP-A, Arp-CBP-B, and Arp-CBP-C, and SGEGs are important shell proteins; role in development; protection from UV radiation and other stressors
Trehalose	Non-reducing disaccharide α-D-glucopyranosyl-1-1-α-D-glucopyranoside; prominent in many anhydrobiotic organisms; diapause-specific in *Artemia*; protects proteins and membranes during desiccation by water replacement and/or vitrification; digested by trehalase; trehalase gene regulated by ArTAP; energy source
LEA proteins	Groups 1 and 3 LEA proteins are found in *Artemia*; unstructured proteins; prominent in many anhydrobiotic organisms; occur in various cell locations; important in mitochondria; diapause-specific in *Artemia*; protect proteins and membranes by molecular shielding and/or vitrification during desiccation
Molecular chaperones	sHSPs and artemin; ATP-independent; diapause-specific; very abundant; protect proteins from irreversible denaturation even when ATP is limiting; mediate tolerance to desiccation and freezing; influence embryo development

amounts of intracellular ferritin were synthesized and used as a chaperone. Artemin oligomer formation, thermostability, and chaperoning depend on cysteine residues (Hu et al. 2011), but there is no correlation between these characteristics. For example, changing C172 maximally reduces chaperone activity but has minimal influence on stability, whereas modifying C22 and C61 has the greatest impact on stability and the least on chaperoning. Cysteine substitutions, along with 1-aniline-8-naphthalene-sulfonate (ANS) reactivity, indicate that surface hydrophobicity contributes to artemin chaperoning (Hu et al. 2011; Shahangian et al. 2011).

Diapause-destined *Artemia* embryos synthesize a wealth of p26 and artemin before release from females, yielding a large supply of molecular chaperones within cysts and signifying their importance in protecting proteins during stress. These chaperones are synthesized prior to diapause and quiescence, not in response to adverse environmental conditions, and they are active in resisting physiological and environmental stressors encountered by cysts.

Summary

Artemia cysts employ an array of protective measures which advance their survival in unforgiving habitats characterized by anoxia, heat, cold, desiccation, UV radiation, and food deprivation (Table 1). The origins and ultrastructure of the cyst shell have been analyzed, with recent experiments revealing the molecular composition of the shell and how the shell contributes selective permeability and resistance to UV radiation. Trehalose is enriched in diapause and quiescent cysts, protecting membranes and proteins from stress-induced damage and serving as a nutrient source for post-diapause growth. Trehalose and LEA proteins are thought to have an important role in protecting against desiccation, perhaps as partners in vitrification which promotes macromolecular stability. p26 and artemin, functioning as ATP-independent molecular chaperones, bolster cyst tolerance to desiccation, freezing, and heat, undoubtedly by preventing irreversible protein denaturation during stress. Moreover, these proteins influence embryo development, diapause termination, and cyst release from females, unexpected functions that expand their significance beyond that related to stress protection. *Artemia* cysts in either diapause or quiescence possess an array of elements that individually protect embryos from physiological and environmental stressors and which when expressed concurrently place *Artemia* among the most stress tolerant of animals.

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