Embryos of the crustacean, *Artemia franciscana*, undergo alternative developmental pathways, producing either larvae or encysted embryos (cysts). The cysts enter diapause, characterized by exceptionally high resistance to environmental stress, a condition thought to involve the sHSP (small heat-shock protein), p26. Subtractive hybridization has revealed another sHSP, termed ArHsp21, in diapause-destined *Artemia* embryos. ArHsp21 shares sequence similarity with p26 and sHSPs from other organisms, especially in the α-crystallin domain. ArHsp21 is the product of a single gene and its synthesis occurred exclusively in diapause-destined embryos. Specifically, ArHsp21 mRNA appeared 2 days post-fertilization, followed 1 day later by the protein, and then increased until embryo release at day 5. No ArHsp21 protein was detected in embryos developing directly into larvae, although there was a small amount of mRNA at 3 days post-fertilization. The protein was degraded during post-diapause development and had disappeared completely from second instar larvae. ArHsp21 formed large oligomers in encysted embryos and transformed bacteria. When purified from bacteria, ArHsp21 functioned as a molecular chaperone *in vitro*, preventing heat-induced aggregation of citrate synthase and reduction-driven denaturation of insulin. Sequence characteristics, synthesis patterns and functional properties demonstrate clearly that ArHsp21 is an sHSP able to chaperone other proteins and contribute to stress tolerance during diapause. As such, ArHsp21 would augment p26 chaperone activity and it may also possess novel activities that benefit *Artemia* embryos exposed to stress.

**Key words:** *Artemia franciscana*, diapause, molecular chaperone, protein structure/function, small heat-shock protein (sHSP), stress tolerance.

### INTRODUCTION

Diapause is a widespread physiological condition where biochemical and morphological changes yield organisms exhibiting decreased growth rate and enhanced stress resistance [1–3]. As an example, embryos of the Branchiopod crustacean *Artemia franciscana* develop either ovoviviparously or oviparously, leading, respectively, to swimming larvae and diapause-destined encysted gastrulae (cysts) [4]. *Artemia* embryo diapause is characterized by interruption of development, and metabolism is reduced so much that it is difficult to detect it [5]. Cysts survive extreme physiological stress including exposure to heat, desiccation and anaoxia, the latter for several years [5,6]. Stress tolerance is thought to depend on the formation of a chitinous wall permeable only to water and volatile substances [7], trehalose [8] and the abundant accumulation of molecular chaperones such as the sHSP (small heat-shock protein) p26 [4,9,10] and the ferritin homologue artemin [11,12]. Like other sHSPs [13], p26 prevents protein denaturation as determined by *in vitro* turbidimetric experiments, confers stress resistance on transfected mammalian cells and inhibits apoptosis [14–19]. Artemin also acts as a molecular chaperone *in vitro* and protects transfected cells [11], in addition to binding and perhaps chaperoning RNA [20]. Developmental regulation, resulting in stage- and tissue-specific sHSP synthesis, occurs in organisms other than *Artemia*, including *Drosophila* [21], *Caenorhabditis elegans* [22,23], *Brugia malayi* [24], *Xenopus laevis* [25] and *Arabidopsis thaliana* [26]. Moreover, sHSP production has been observed during diapause in some [2,27,28], but not all [29,30], organisms.

A subtractive hybridization study was undertaken previously to investigate molecular changes associated with diapause induction and maintenance in *Artemia* embryos [31,32]. Among the up-regulated transcripts observed for the first time in diapause-destined embryos was mRNA for the sHSP ArHsp21. The ArHsp21 cDNA sequence is shown in the present study to encode an α-crystallin domain typical of sHSPs. ArHsp21 mRNA was detected in diapause-destined *Artemia* embryos at 2 days post-fertilization and increased until cyst release. In contrast, ArHsp21 transcripts rose transiently in ovoviviparous embryos and had all but disappeared when larvae were discharged. ArHsp21 protein was detected only in diapause-destined embryos and it vanished during larval development. Purified ArHsp21 formed high-molecular-mass oligomers and functioned as a molecular chaperone *in vitro*. The findings demonstrate that ArHsp21 is similar to other sHSPs, suggesting that it contributes, together with p26 and artemin, to the enhanced stress tolerance characteristic of encysted *Artemia* embryos.

### EXPERIMENTAL

**Artemia** culture and embryo preparation

*A. franciscana* cysts (INYE Aquaculture, Ogden, UT, U.S.A.) were hydrated overnight at 4°C in distilled water, rinsed three times in cold water and incubated in Hatch medium [33] at 27°C for 20 h with shaking at 200 rev/min. Synchronous emerged nauplii (E1) were homogenized or allowed to develop into first and second instar larvae [9,34], which in turn were either homogenized or incuated continuously at room temperature (18°C) in sea
water with *Isochrysis galbana* (clone synonym: ISO) or *Isochrysis* sp. (clone synonym: TISO) (Provasoli-Guillard Center for Culture of Marine Phytoplankton, West Boothbay, ME, U.S.A.). Adult *Artemia* were maintained at room temperature in batch culture, or individual females with oocyte-filled lateral pouches were placed in 6-well plates with males for fertilization. Embryos were collected from ovisacs at 1-day intervals post-fertilization and frozen in liquid nitrogen.

**ArHsp21 cDNA cloning and sequencing**

RNA (10 µg) prepared with TRIzol® (Invitrogen, Burlington, ON, Canada) from diapause-destined *Artemia* embryos at 2 days post-fertilization was incubated at 37°C for 1 h with calf intestine alkaline phosphatase (Ambion, Austin, TX, U.S.A.) and purified with acid phenol/chloroform. The RNA was treated with tobacco acid pyrophosphatase (Ambion) at 37°C for 1 h and then ligated with the 5′-RACE (5′-rapid amplification of cDNA ends) adaptors (5′-GGCUGAGGGAUGAACACUCGCUUUGCGGUUUGAAGAA-3′) using T4 RNA ligase. The ligated RNA was reverse-transcribed to cDNA at 42°C for 1 h in a mixture of random decamers, MMLV (Moloney-murine-leukaemia virus) reverse transcriptase (Ambion) and dNTP. Nested PCR was performed using the 5′-RACE outer primer 5′-GCTGATGGCGATGAATGAACACTG-3′ and the inner primer 5′-CGCGGATCCGAACACTGCGTTGCTGGCTTTGATG-3′ together with the ArHsp21 outer primer 5′-GTCTAAGAATATCAGTCTCTCGTGGT-3′ and the inner primer 5′-GCAAGCTTGATTCGTTTCCGATCC-3′, the last two sequences being based on subtractive hybridization data [31]. The 5′-RLM-RACE PCR conditions were 94°C for 3 min and then 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min and 72°C for 7 min. The PCR products were inserted into a TA cloning vector (Invitrogen), transformed into TOPO10 Competent *E. coli* (Invitrogen) and sequenced (DNA Sequencing Facility, Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada). The ArHsp21 amino acid sequence was derived from cDNA by Gene Runner.

**Detection of the ArHsp21 gene on Southern blots**

ArHsp21 cDNA was digoxigenin-11-dUTP-labelled by using the PCR Dig Labelling Mix (Roche, Mannheim, Germany) from cDNA, ethanol-precipitated, dissolved in 20 µl of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5 with HCl) and electrophoresed in 0.7% agarose gel. Gels were immersed twice for 15 min in denaturation solution (0.5 M NaOH and 1.5 M NaCl) followed by two incubations in neutralization solution (0.5 M Tris/HCl, pH 7.5, and 1.5 M NaCl) for 15 min and one in 20 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) for 10 min. DNA was transferred to nitro membranes (Roche, Mannheim, Germany) in transfer buffer (Ambion), which were washed with 2 × SSC, baked at 80°C for 2 h, prehybridized in Dig Easy Hyb (Roche) at 42°C for 6 h and hybridized overnight with probes in Dig Easy Hyb at 46°C. The membranes were washed twice at room temperature in 2 × SSC containing 0.1% SDS and twice at 68°C in 0.5 × SSC with 0.1% SDS prior to incubation with CDP-Star (Roche) and exposure to an X-ray film (Fuji Photo Film Co., Tokyo, Japan).

**Quantification of ArHsp21 mRNA by real-time PCR**

Embryo RNA was prepared with TRIzol® (Invitrogen) at daily intervals from day 0 to 5 post-fertilization, genomic DNA was removed with the TURBO DNA-free kit (Ambion) and RNA was reverse-transcribed with the First-strand cDNA Synthesis kit (Amersham Biosciences). PCR in the iCycler (Bio-Rad, Mississauga, ON, Canada) was in 25 µl mixtures containing 0.5 µl of cDNA, 12.5 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), 0.5 µl of Rox reference dye (Invitrogen) and 50 ng of the forward primer 5′-AGACCCAAATCCGTCACCTGTGGTTT-3′ and the reverse primer 5′-ATCAATCTTGCAGCTCCTCGGCTT-3′. Reaction conditions were 50°C for 2 min, 95°C for 2 min, 45 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s, followed by 72°C for 5 min. α-Tubulin was amplified with the forward primer 5′-CTGCACTGCTGCTAGAGGAGATGTG-3′ and the reverse primer 5′-CTCCTCTCAAGAGTCCATGCCA-3′.

**ArHsp21 purification and antibody production**

RT–PCR (reverse transcription–PCR) was performed with Pfu DNA polymerase (Fermentas Life Sciences, Burlington, ON, Canada) using RNA from oviparous embryos in conjunction with the ArHsp21 forward primer 5′-GCTTCCGAGATGCTAGTGATGACCT-3′ and the reverse primer 5′-GCAAGCTTGAATATCAGTCTCTCGT-3′, containing a HindIII site, and the reverse primer 5′-CACGTTGAATATCAGTCTCTCGT-3′, containing a HindIII site. After adenine attachment with an A-Addition kit (Qiagen, Mississauga, ON, Canada), PCR products were cloned into a TA vector (Invitrogen) and transformed into TOP10 competent *E. coli* (Invitrogen). Plasmid DNA was purified, digested with Xhol and HindIII and resolved in agarose gels prior to cDNA insert recovery for ligation into the His-tagged expression vector pRSET A (Invitrogen) and transformation into TOP10 F′ *E. coli*. The cDNA insert was sequenced and transformed into E. coli BL21 (DE3) pLysS (Invitrogen).

For ArHsp21 synthesis, transformed bacteria were induced for 6 h with 1 mM IPTG (isopropyl β-D-thiogalactoside; Sigma, Oakville, ON, Canada), harvested by centrifugation at 2000 × g for 15 min at 4°C, washed with PBS and resuspended in Equilibration/Wash buffer (BD Biosciences, Mississauga, ON, Canada) containing protease inhibitors [36] and lysozyme (Sigma) at 0.75 mg/ml. Mixtures were incubated at room temperature for 20 min, frozen and thawed three times, and sonicated three times for 10 s with intermittent cooling on ice for 30 s. The lysate was centrifuged at 12000 × g for 10 min and the supernatant was incubated for 20 min with BD TALON metal-affinity resin (BD Biosciences). The resin was washed twice with 15 bed volumes of a buffer containing 10 mM imidazole, 50 mM sodium phosphate and 500 mM NaCl (pH 7.5), packed in a column, washed with 10 bed volumes of the buffer and eluted with Elution Buffer (BD Biosciences). Samples containing ArHsp21 were dialysed in 10 mM phosphate buffer (pH 7.2) for 4 h at room temperature with one buffer change and then overnight at 4°C, followed by concentration in Microcon YM-10 centrifugal filter devices (Millipore, Bedford, MA, U.S.A.).

Purified ArHsp21 was emulsified with TiterMax™ Gold Adjuvant (Sigma), injected subcutaneously into rabbits three times at 15-day intervals and serum was harvested 45 days after the first injection. Rabbits, from Charles River Canada (St. Constant, QC, Canada), were cared for in accordance with the guidelines in the *Guide to the Care and Use of Experimental Animals*.
Figure 1  Alignment of ArHsp21 with other sHSPs

(A) The amino acid sequences of ArHsp21 and Artemia p26 (AF031367) were aligned by using ClustalW. (B) The amino acid sequence of ArHsp21 was aligned by using ClustalW with Artemia p26, human α-crystallin (h-A) (P02489), human αB-crystallin (h-B) (P02511), wheat HSP16.9 (WHSP16.9) (S21600) and Drosophila hsp26 (Drohsp26) (AAA28636). N-terminal regions, α-crystallin domains and C-terminal extensions are indicated. Shared motifs and a highly conserved arginine in the α-crystallin domain are boxed. ‘*’, Identical residues; ‘:’, conserved substitution; ‘.’, semi-conserved substitution.

Animals available from the Canadian Council on Animal Care. To test antibody activity, blots containing bacterially produced ArHsp21 were probed with primary antibodies, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunologicals, West Grove, PA, U.S.A.). Protein detection was with Enhanced Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA, U.S.A.) and an X-ray film.

Detection of ArHsp21 during Artemia development

Developing embryos were collected at daily intervals for 5 days post-fertilization, rinsed in Hatch medium and homogenized in 0.4 ml of TRIzol® (Invitrogen). Homogenates were incubated for 5 min at room temperature, followed by addition of 80 μl of chloroform and centrifugation at 12 000 g for 15 min at 4°C. The upper protein-containing phase was mixed with 120 μl of ethanol, incubated at room temperature for 3 min and centrifuged at 2000 g for 5 min at 4°C. Supernatants were incubated with 600 μl of propan-2-ol for 10 min at room temperature and centrifuged at 12 000 g for 10 min at 4°C. Pellets were washed three times in 95% (v/v) ethanol containing 0.3 M guanidinium chloride, once in ethanol and then dissolved in 30 μl of 1% SDS. Encysted Artemia embryos, emerged nauplii enclosed in hatching membranes (EIII) [34], first and second instar larvae and adult males were also harvested, washed with Hatch medium and homogenized on ice for 10 min in Pipes buffer (100 mM Pipes, 1 mM MgCl₂ and 1 mM EGTA, pH 6.5) with protease inhibitors [36]. Homogenates were centrifuged at 12 000 g for 10 min at 4°C and supernatants were transferred to fresh tubes prior to centrifugation for 5 min. All the protein samples were subjected to SDS/12.5% PAGE and either stained with Coomassie Brilliant Blue or blotted on to nitrocellulose and probed with antibodies.
ArHsp21 oligomerization

A 400 μl portion of ArHsp21-containing protein extracts from bacteria and Artemia cysts was loaded individually on to 1 ml of 10–50% continuous sucrose gradients in 0.1 M Tris/glycine (pH 7.4) and centrifuged at 40000 rev./min for 16 h at 4°C in a Beckman SW41 T1 rotor. Tubes were fractionated and samples were subjected to SDS/PAGE for blotting and immunodetection with Omni-probe (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Molecular-mass markers of 669 kDa (thioglycolobin), 443 kDa (apoferritin), 200 kDa (α-amylase), 66 kDa (BSA) and 29 kDa (carbonic anhydrase) (Sigma) were centrifuged in gradients and fractionated and the A280 (absorbance at 280 nm) was measured.

ArHsp21 chaperone activity

Citrate synthase (Sigma) at 150 nM was mixed with purified ArHsp21 at concentrations of 37.5, 75, 150, 300 and 600 nM in 40 mM Hepes/KOH buffer (pH 7.5) in 1 ml reactions and heated at 43°C with monitoring at 360 nm. Protection against reduction-induced protein denaturation was measured in 300 μl reaction mixtures containing 4 μM insulin, 20 mM DTT (dithiothreitol) and purified ArHsp21 at 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 μM. Protein aggregation was measured at 400 nM in 96-well plates at 25°C with a SPECTRAmax PLUS spectrophotometer (Molecular Devices). BSA (Sigma) and bovine immunoglobulin (IgG) (Sigma) replaced sHSPs in control assays.

RESULTS

ArHsp21 is an sHSP encoded by a single gene

A full-length ArHsp21 cDNA (accession number DQ361273), including an open reading frame of 546 bp, a 77 bp 5′-UTR (5′-untranslated region) beginning at the transcription start site, a 129 bp 3′-UTR containing a polyadenylation site and a poly(A) tail, was cloned. The deduced ArHsp21 amino acid sequence (accession number ABD19712) consisted of 181 residues with a molecular mass of 21.1 kDa. ArHsp21 shares 29% identity and 51% similarity with p26, another Artemia sHSP (Figure 1A). A highly conserved arginine occurs in the ArHsp21 α-crystallin domain and a V/I/X/V motif in the C-terminal extension, common sHSP features (Figure 1B). Southern blots containing Artemia DNA probed with labelled ArHsp21 cDNA gave single bands of approximately 9.1, 8.8 and 4.0 kb when digested, respectively, with BamHI, SacI and XhoI, indicating one ArHsp21 gene in the Artemia genome.

ArHsp21 synthesis is diapause-specific

ArHsp21 mRNA increased in diapause-destined Artemia embryos, reaching a peak just prior to cyst release at day 5 post-fertilization (Figure 2). In contrast, ArHsp21 mRNA increased from fertilization to day 3 in embryos developing into nauplii, although to a lesser extent than in diapause-destined embryos, and then returned to low levels (Figure 2). In order to prepare antibody, ArHsp21 and p26 were synthesized in transformed bacteria and purified (Figures 3A–3D). A strong ArHsp21 band was visible in Coomassie Brilliant Blue-stained gels, whereas p26 production was lower, with His-tagged ArHsp21 and p26 at 28 and 32 kDa respectively. Antibodies to ArHsp21 and p26 recognized the corresponding protein with no cross-reactivity (Figures 3E–3G). ArHsp21 was first detected in protein extracts of diapause-destined embryos at 3 days post-fertilization and persisted until cyst release (Figures 4A and 4B), but it was missing from extracts of embryos developing into nauplii (Figures 4C and 4D). ArHsp21 was detected in post-diapause cysts and in emerged larvae (EIII) and first instar larvae arising from cysts, but not in second instar larvae and adults, indicating proteolytic degradation (Figures 4E and 4F).

ArHsp21 oligomerization and molecular chaperone activity

As revealed by sucrose-density-gradient centrifugation, the maximal mass of ArHsp21 oligomers in cell-free extracts from transformed bacteria was somewhat smaller than for oligomers from cysts (Figures 5A and 5B). Additionally, cyst extracts contained more monomeric ArHsp21 than did bacterial extracts. Purified ArHsp21 inhibited heat-induced citrate synthase denaturation and reduction-dependent insulin aggregation in a concentration-dependent manner, whereas BSA and bovine immunoglobulin had little effect (Figures 6A–6D). Oligomerization and chaperoning were tested on two independent samples, with similar results in duplicate experiments for each preparation.

DISCUSSION

ArHsp21, the second sHSP identified in diapause-destined Artemia embryos, consists of an N-terminal region, an α-crystallin...
Diapause-specific small heat-shock protein in Artemia embryos

Figure 4  ArHsp21 synthesis and degradation are developmentally regulated

Protein extracts from Artemia embryos at daily intervals post-fertilization were subjected to SDS/PAGE and either stained with Coomassie Brilliant Blue (A, C) or blotted on to nitrocellulose and stained with antibodies directed to ArHsp21 (B, D). (A, B) Diapause-destined embryos; (C, D) embryos developing into larvae. Lane 1, day 0; lane 2, day 1; lane 3, day 2; lane 4, day 3; lane 5, day 4; lane 6, day 5. Each lane was loaded with 30 µg of protein. (E, F) Protein extracts from post-diapause developing Artemia were subjected to SDS/PAGE and either stained with Coomassie Brilliant Blue (E) or transferred on to nitrocellulose and probed with antibodies to ArHsp21 (F). Each lane was loaded with 50 µg of extract. Lane 1, undeveloped cysts; lane 2, emerged larvae; lane 3, first instar larvae; lane 4, second instar larvae; lane 5, adult males; lane M, molecular-mass markers in kilodaltons.

Figure 5  ArHsp21 oligomerization

Protein extracts from bacteria (A) and encysted embryos (B) were centrifuged in 10–50% continuous sucrose gradients prior to fractionation and ArHsp21 detection on Western blots. Molecular-mass markers in kilodaltons are indicated by labelled arrows.

domain and a C-terminal extension, as is common for molecular chaperones of this type. The N-terminal region of ArHsp21 contains a WPDF motif, which also appears in p26, an extensively characterized, diapause-specific sHSP found in several Artemia species [19,35]. This motif occurs in other sHSPs, including those from mammals, where it influences oligomerization and chaperoning in vitro, possibly via intramolecular interactions with the α-crystallin domain [13,37]. ArHsp21 possesses an arginine at position 123 corresponding to the most highly conserved residue in the sHSP α-crystallin domain [13]. When Arg114, the equivalent p26 residue, is modified to alanine by site-directed mutagenesis, chaperone activity decreases substantially [14], as happens for similar changes in mammalian sHSPs [38] that lead to disease [39–41]. The conserved C-terminal motif I/VXV/I, which is thought to stabilize oligomer structure by establishing intersubunit interactions [42], exists in ArHsp21 as 161PI163, demonstrating similarity to other sHSPs in this regard. ArHsp21 from Artemia embryos assembles into oligomers as large as 669 kDa, somewhat greater in mass than those in protein extracts from transformed bacteria. By comparison, lens α-crystallin oligomers range from 300 kDa to 1 MDa [43], Hsp27 mass varies from 200 to 800 kDa [44] and Hsp20 exists in a concentration-dependent equilibrium between 43 and 470 kDa [45]. Functionally, sHSPs prevent irreversible aggregation of denaturing proteins, serving as the first line of defence when cells are stressed. In this vein, ArHsp21 inhibits heat-induced citrate synthase aggregation and

Figure 6  ArHsp21 has molecular chaperone activity

(A) Purified ArHsp21 was incubated with 150 nM citrate synthase at 43°C and turbidity increases were measured at 360 nm. Curve 1, no ArHsp21; curve 2, 37.5 nM; curve 3, 75 nM; curve 4, 150 nM; curve 5, 300 nM; curve 6, 600 nM. (B) Curve 1, no ArHsp21 added; curve 2, 600 nM bovine immunoglobulin; curve 3, 600 nM BSA. (C) Purified ArHsp21 was incubated with 4 µM insulin at 25°C and attenuance was measured at 400 nm on addition of DTT. Curve 1, no ArHsp21; curve 2, 0.1 µM; curve 3, 0.2 µM; curve 4, 0.4 µM; curve 5, 0.8 µM; curve 6, 1.6 µM. (D) Curve 1, no ArHsp21; curve 2, 1.6 µM bovine immunoglobulin; curve 3, 1.6 µM BSA.
DTT-driven insulin denaturation, commonly used indicators of molecular chaperone capability in vitro. ArHsp21 and p26 [14,15] are equally effective as molecular chaperones, and the activities of both proteins are concentration-dependent, with elevation of sHSP increasing chaperone capacity in vitro. ArHsp21 is clearly a typical sHSP with the potential to protect proteins in cells exposed to stress.

Probing of Southern blots containing restriction-digested genomic DNA from *Artemia* implied a single ArHsp21 gene lacking introns, or at least introns lacking recognition sequences for the enzymes tested. In contrast, the p26 gene, previously sequenced in its entirety, contains three introns, one of which localizes to the 5'-non-coding region [35]. These results suggest substantial differences between ArHsp21 and p26 genes in spite of the conserved features evident in their protein products. Intron composition varies for other sHSP genes. *Drosophila* sHSP genes lack introns except for one in *l(2)efl* [46], and the four Hsp16 genes from *C. elegans* each possess a single intron, as does the SEC-1 gene, with only Hsp12.3 containing two introns [23,46,47]. Sequencing of the ArHsp21 gene is required to complete the comparison with p26, the only crustacean sHSP gene for which structural analysis is available. Of particular interest are sequences and intron compositions, characteristics that may illuminate *Artemia* sHSP gene evolution.

The appearance of ArHsp21 mRNA at 2 days post-fertilization, followed by accumulation in diapause-destined *Artemia* embryos, as opposed to the minor transient rise in transcript content in embryos giving rise directly to larvae, demonstrates developmental stage at which diapause manifests, the level of embryos giving rise directly to larvae, demonstrates development and suggests that each protein contributes differently to diapause physiology. By comparison, sHSP accumulation characterizes diapause in the brine-fly, among other insects [27,28], and the nematode *C. elegans* [2] but not in the blow-fly [29] or in the fruitfly, *Drosophila triauraria* [30]. It is not clear why such differences exist, but they may be related to the developmental stage at which diapause manifests, the level of stress tolerance attained and/or the degree of metabolic decline, with the last two characteristics reaching extreme limits in *Artemia* embryos. Interestingly, seeds have many characteristics in common with *Artemia* cysts, including resistance to desiccation and other stressors. Plants possess a large number of sHSPs, some appearing in seeds under developmental control in the absence of stress and then declining upon germination [48,52,53]. These proteins may contribute to stress tolerance, as is thought to be the case in diapause *Artemia*.

To summarize, *Artemia* embryo encystment and diapause are integrated developmental programmes requiring the coordinated synthesis and function of many molecules. Among these are the sHSPs including p26, a well-characterized molecular chaperone, and now ArHsp21, both of which oligomerize and possess chaperone activity. Like p26, ArHsp21 build-up is developmentally regulated, likely controlled at transcription in embryos and by proteolysis during post-diapause growth. The results corroborate the hypothesis that sHSPs are required for the initiation and maintenance of diapause in *Artemia* embryos. However, the numbers of these proteins and their functional relationships during diapause are yet to be determined definitively for *Artemia*, or any other organism, thus representing an intriguing area of investigation with both fundamental and applied significance.

This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council of Canada and a Grant-in-Aid from the Heart and Stroke Foundation of Nova Scotia to T.H.M.

**REFERENCES**


© The Authors Journal compilation © 2008 Biochemical Society


423