Habitat diversity and adaptation to environmental stress in encysted embryos of the crustacean *Artemia*

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Encysted embryos (cysts) of the brine shrimp, *Artemia*, provide excellent opportunities for the study of biochemical and biophysical adaptation to extremes of environmental stress in animals. Among other virtues, this organism is found in a wide variety of hypersaline habitats, ranging from deserts, to tropics, to mountains. One adaptation implicated in the ecological success of *Artemia* is p26, a small heat shock protein that previous evidence indicates plays the role of a molecular chaperone in these embryos. We add to that evidence here. We summarize recently published work on thermal tolerance and stress protein levels in embryos from the San Francisco Bay (SFB) of California inoculated into experimental ponds in southern Vietnam where water temperatures are much higher. New results on the relative contents of three stress proteins (hsp70, artemin and p26) will be presented along with data on cysts of *A. tibetiana* collected from the high plateau of Tibet about 4·5 km above sea level. Unpublished results on the stress protein artemin are discussed briefly in the context of this paper, and its potential role as an RNA chaperone. Interestingly, we show that the substantial tolerance of *A. franciscana* embryos to ultraviolet (UV) light does not seem to result from intracellular biochemistry but, rather, from their surrounding thick shell, a biophysical adaptation of considerable importance since these embryos receive heavy doses of UV in nature.

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1. Introduction

Species of the brine shrimp, *Artemia*, are found in a variety of harsh environments in many parts of the world (Triantaphyllidis *et al* 1998; Van Stappen 2002) where they encounter severe hypersalinity, high doses of ultraviolet radiation, very low oxygen tensions and extremes of temperature (Persoone *et al* 1980; Decleir *et al* 1987; MacRae *et al* 1989; Warner *et al* 1989; Browne *et al* 1991; Abatzopoulos *et al* 2002). These challenging ecological settings make *Artemia* a useful model organism for studies on evolutionary and ecological aspects of the stress response, at all levels of biological organization (Clegg and Trotman 2002).

The stress (heat shock) response has been the object of intense study (for example, Morimoto *et al* 1994; Fiege *et al* 1996; Feder and Hofmann 1999; Arrigo and Müller 2002). One feature of the heat shock response is the contribution from an ensemble of heat shock (stress) protein families, many being molecular chaperones. The literature is massive (see references above, and Beissinger and Buchner 1998; Lorimer and Baldwin 1998; Richardson *et al* 1998; Ellis 1999; Ellis and Hartl 1999; Fink 1999; van den IJssel *et al* 1999).

Current research on the stress response is aimed chiefly at the cellular and molecular levels but, as Feder and Hofmann (1999) point out, the evolutionary and ecological aspects have been given increased recent attention

Keywords. Anoxia; *Artemia*; heat shock; stress proteins; ultraviolet radiation

Abbreviations used: dd, Distilled deionized; ISA, International Study of *Artemia*; SFB, San Francisco Bay; UV, ultraviolet.

(see Feder and Block 1991; Coleman *et al* 1995; Norris *et al* 1995; Hightower 1998; Clegg *et al* 1999; Krebs 1999; Tomanek and Somero 1999). We believe that *Artemia* can be used to great advantage in that regard, an opinion that motivated some of the studies to be summarized here.

Most experimental research on *Artemia* has used *A. franciscana* which occurs naturally, and primarily in the Great Salt Lake, Utah, and in salterns located in the South San Francisco Bay (SFB), although many other populations exist, some the result of human intervention. For example, since 1987 *A. franciscana* has been grown during the dry season in southern Vietnam (Baert *et al* 1997; Hoa 2000). Because ambient temperatures are much higher in these ponds, compared to SFB salterns, it seemed worthwhile to compare cysts from SFB with those produced in Vietnam to determine whether the latter have acquired enhanced resistance to higher temperatures. We note that of all *Artemia* species examined, *A. franciscana* seems to exhibit the highest level of phenotypic plasticity (Browne and Wanigasekera 2000) and this versatility endows this species with certain virtues when it comes to using it as an inoculum for culture in locations where *Artemia* does not exist naturally.

In previous studies (Clegg *et al* 2001) we compared the thermal tolerance of cysts produced by sequential inoculation of SFB cysts in the Vietnam culture ponds during the years 1996, 1997 and 1998. That study examined the stress proteins in the 1998 population, but not the other two years. In the results to be presented here we extend the coverage to include an analysis of the cysts produced during the first growing season (1996). We also compare these results to stress proteins in the cysts of *A. tibetiana* from the Tibet high plateau, a very cold habitat (Clegg *et al* 2001).

Finally, we show that the outer layer of the shells of *A. franciscana* cysts is of major importance in protecting them from ultraviolet irradiation, a stress that we believe all species of *Artemia* encounter in nature.

2. Materials and methods

2.1 *Sources of* Artemia *embryos and decapsulation*

A. franciscana from salterns in the SFB were purchased from San Francisco Bay Brand, Hayward, CA, as dried (activated) encysted embryos, and stored at about – 10°C under 100% N_2. Embryos were placed at room temperature for 5 days before use, and had a final hatching percentage close to 90%. Dried embryos were hydrated in seawater overnight at 2°C to suppress metabolism before study. In some cases the outer shell was removed (a process called decapsulation) prior to studies on exposure to ultraviolet light (UV). Briefly, 5 g hydrated embryos were washed well with ice-cold distilled deionized (dd) H_2O, blotted, and placed in 200 ml of antiformin (7% NaOH, 3% Na_2CO_3, 1% NaCl in 50% v/v Clorox™ bleach) on ice for 20 min, with frequent stirring. When the outer shell dissolved and the inner compartment (embryos) became visible, 200 ml of ddH_2O were added to dilute the antiformin which was decanted. These embryos were washed twice with 400 ml ice-cold 0·5 M NaCl, followed by 5 min of gentle stirring in 300 ml 1% sodium thiosulphite, rinsing with ddH_2O, then 5 min of gentle stirring in 300 ml 0·1 M HCl, and a final wash in ddH_2O. These steps neutralized and removed adsorbed hypochlorite. Decapsulated embryos were used immediately or stored at 2°C in 0·5 M NaCl overnight and then used. Hatching was monitored after each decapsulation to ensure that hatch level was not affected. Cysts of *A. tibetiana* were a kind gift from Professor Zheng Mianping, Chinese Academy of Geological Sciences, Beijing, PR China, via the Artemia Reference Center, Gent, Belgium. These cysts were obtained from Lagkor Lake, hypersaline (91 ppt) and carbonate-based, located on the high plateau of Tibet (84°E, 32°03′N) at 4,490 m elevation where the average yearly water temperature is 1–2°C. Further, details on this new species have been published (Zunying *et al* 1998; Van Stappen *et al* 2003; Abatzopoulos *et al* 1998).

2.2 *Hatching assays and developmental biology*

Groups of at least 200 embryos were placed into 20-welled plastic depression plates, each well containing 10–20 embryos in 300 µl of 0·2 mm-filtered seawater. The plates were covered, sealed with tape to prevent evaporation, and incubated in constant light at 21–24°C until hatching was complete. Adequate O_2 is present in these sealed plates since controls (activated embryos) exhibited 88–91% hatching within 2 days. All methods used to study the development of embryos in the female ovisac have previously been described in detail (Jackson and Clegg 1996) including their removal for histology and analysis for the stress proteins p26 and artemin.

2.3 *Heat shock and anoxia*

Embryos were heated in aerated seawater in a Lauda RM40 water bath (accurate to ± 0·05°C), at a rate of 0·7°C/min, from 22°C to 50°C, then used at once for analysis, or incubated further at 50°C. The temperature of 50°C was selected because this translocates the maximum amount of p26 into nuclei without killing any embryos (Clegg *et al* 1999). Embryos were made anoxic with 100% N_2 using procedures described in detail previously (Clegg 1997).

2.4 *Analysis of p26 and artemin in heat-shocked and previously anoxic embryos*

Embryos that had been heat-shocked or had previously undergone 1 month and 1 year of anoxia were homogenized in buffer K (5 mM $MgSO_4$, 5 mM HaH_2PO_4, 40 mM Hepes, 70 mM potassium gluconate, 150 mM sorbitol, pH 6·5). In some cases the homogenates were analysed directly, while in others the nuclei and yolk platelets were removed (1600 g, 5 min) and the supernatant applied to a column of Sepharose CL-6B, calibrated with several native proteins of known molecular weight. Fractions were obtained, reduced to equal volumes under vacuum and then analysed by SDS-PAGE with protein detection by Coomassie staining and p26 by Western blotting. Further, details about all these methods are given in Clegg *et al* (1994).

2.5 *SDS-PAGE and immunoblotting*

Known volumes of homogenates and other fractions were added to equal volumes of 2X sample buffer (Laemmli 1970) and heated at 100°C for 5 min. After low speed centrifugation (1600 g, 5 min) to remove shell fragments, supernatants were electrophoresed in 12% polyacrylamide gels, and proteins detected by Coomassie blue-G. Proteins from SDS-PAGE were also transferred to nitrocellulose sheets and prepared for immunodetection using a polyclonal anti-p26 and anti-artemin at 1 : 5,000 for 1 h (Clegg *et al* 1994) as the primary antibodies, and horseradish peroxidase conjugated anti-rabbit IgG (1 : 5,000, 1 h) as secondary. For the detection of hsp70, primary antibody was purchased from Affinity BioReagents, Inc. (MA3-001; 1 : 1,250, 2 h) and the secondary used was horseradish peroxidase conjugated anti-rat IgG (1 : 5,000, 1 h). Chemiluminescence was detected on X-ray film with Super Signal® West Pico (Pierce, Rockford, Illinois USA).

2.6 *Ultraviolet irradiation*

A Minerallight UVSL 25 lamp, specified for 180 $\mu W/cm^2$ at 15 cm and emitting a wavelength of 254 nm was used. The path length to the specimens was 4·5 cm, so at this distance the lamp is irradiating at 607 $\mu W/cm^2$. The fully hydrated embryos, decapsulated or not, were spread as 'monolayers' on the surface of water-moistened (but not wet) filter paper. Irradiated embryos were assayed for viablility by evaluating the percentage of embryos giving rise to swimming larvae after treatment.

2.7 *The Vietnam experiment*

A. franciscana cysts from the South San Francisco Bay (SFB), USA were obtained from the *Artemia* Reference Center, Gent University, Belgium, as part of the cyst collection and the International Study of *Artemia* (ISA). These cysts (ISA 1258) were used to inoculate growth ponds in Vietnam in the dry season of 1996, and cysts produced by the resulting adults during a single growing season were harvested, dried and stored for later study. This project, referred to here as 'the Vietnam experiment' and performed chiefly by Dr Nguyen Van Hoa (Hoa 2002), was carried out in experimental ponds of the Institute of Marine Aquaculture, Can Tho University, located in the salterns of the Vinh Tien Shrimp-Salt Cooperative, Vin Chau District, in the Mekong Delta of Vietnam (see Baert *et al* 1997). Salinity in the growth ponds ranged from 62–115 ppt, and the water temperatures during the growing season ranged from a low of 24°C to a high of 38°C, essentially on a daily basis. In contrast, water temperatures in the South San Francisco Bay very rarely exceed 20°C at any time during the year.

3. Results

3.1 *General description of* Artemia

Because most of the research to be presented here relies on some understanding of this organism we provide a description based on *A. franciscana* which we believe is similar to all bisexual *Artemia* species, at least in general detail.

Figure 1 is an adult couple in which the encysted embryos (cysts) are barely visible in the female ovisac beneath the male. Bisexual *Artemia* of all species exhibit this coupling behaviour; however, fertilization occurs only after the female molts. Figure 2 shows the structure of a cyst and selected organelles (right panel). The embryo (a gastrula containing about 4000 nuclei) is surrounded by a complex shell, which we will show here provides substantial protection against UV irradiation. In general, these embryos have a structure typical of yolky crustacean embryos, and their ultrastructure provides no obvious insight into the basis of their extraordinary stress resistance.

Figure 3 describes the morphology of embryos undergoing the two modes of development exhibited by most species of *Artemia*. The direct pathway is not interrupted by dormancy, but leads to the production of naupluis larvae that are released from the female. In contrast, the diapause pathway produces an encysted gastrula that enters diapause, a state of obligate metabolic dormancy and developmental arrest. The arrow points to the embryonic cuticle, present in these embryos but not those undergoing direct development.

3.2 *Ontogeny of p26 and artemin*

Accompanying the morphological changes of early development (figure 3) is the appearance of two major proteins,

p26 and artemin, that will be focused on in this paper (figure 4). Artemin lags behind p26 by about a day, but both appear rather late in terms of formation of the diapause gastrula. We stress that neither p26 nor artemin can be detected in embryos undergoing the direct pathway, in spite of considerable effort (Jackson and Clegg 1996 and unpublished results on artemin).

3.3 *Artemin and p26 in heat-shocked and anoxic embryos*

When encysted embryos are heat-shocked, much of the p26 translocates to nuclei (figure 5). In contrast, these data show that artemin remains in the extra-nuclear space ('soluble'). When embryos are heat-shocked and then returned to physiological temperatures, the p26 translocated to nuclei returns to the supernatant fraction, this translocation being complete by about an hour after heat-shock (figure 6).

Figure 7 shows the analysis of control and heat-shocked embryo supernatants (no nuclei present) using Sepharose-CL-6B gel filtration fractionation followed by SDS-PAGE. Note that very little p26 is in the void volume of control supernatants (fractions 1–3) and that little protein of any kind is found there, suggesting minimal protein aggregation (figure 7A, a). In contrast, supernatants from heat-shocked embryos contain appreciable protein in the void volume (fractions 1–3) including significant amounts of p26 (figure 7B, b).

Supernatants analysed similarly from previously anoxic embryos are shown in figure 8. The arrow indicates artemin, being very much the same in embryos undergoing one week or one year of anoxia; that is, anoxia does not

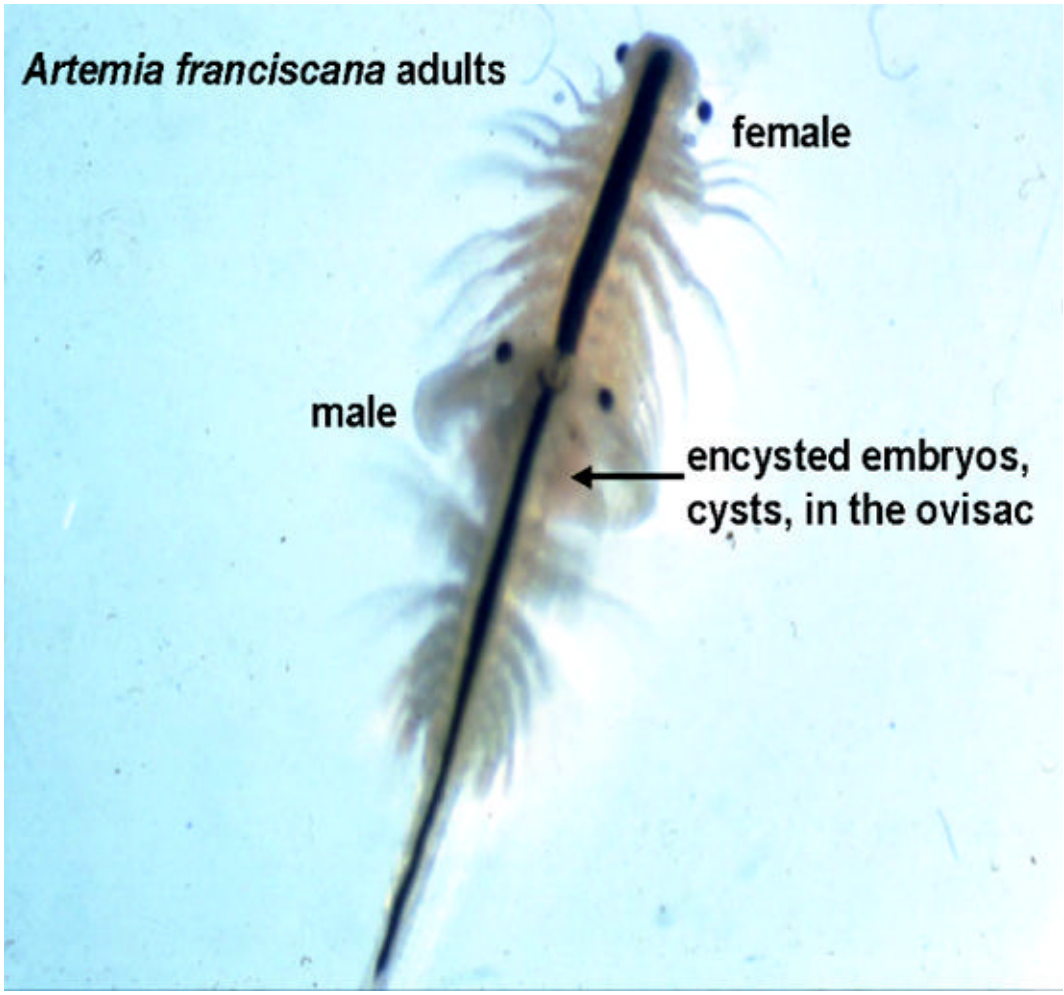

Figure 1. A mature *Artemia* 'couple' with encysted embryos (cysts) barely visible in the ovisac of the female, ventral to the male. The cysts are in developmental arrest upon release, but remain metabolically active for several days (see Clegg and Jackson 1998; Clegg *et al* 1999).

lead to its translocation. The location of p26 is slightly below artemin (whose sub-units are about 28 kDa). However, most of the p26 has undergone translocation to nuclei and other locations during anoxia, and is spread across most of the fractions from the gel filtration column. But the key feature of these results is the striking similarity in the protein profiles, with remarkably little protein aggregation taking place (which would be seen in fractions 1–3) even during a year of continuous anoxia.

3.4 *The Vietnam experiment and* A. tibetiana

Table 1 compares the levels of artemin, p26 and hasp70 in cysts (VN-Y1) produced during a single growing season after the ponds were inoculated with cysts from SFB/ISA. Substantially higher levels of p26, artemin and one of the hsp70 isoforms are present in the cysts produced in Vietnam. On the other hand, all of these proteins are present in much lower amounts in *A. tibetiana* (table 1).

3.5 *Protection against ultraviolet irradiation*

Although the colour of intact cysts of *A. franciscana* varies, they are usually various shades of brown and always opaque, so the underlying embryos cannot be seen. figure 9 shows the morphology of encysted embryos that have had the outer thick layer of their shells removed by a process called 'decapsulation' (§ 2). Although the embryos exhibit a wide range of colouration (the remaining parts of the shell are transparent) the dominant colours are off-white and different degrees of orange (figure 9). When these embryos are exposed to UV irradiation their death rate greatly exceeds that of embryos whose shells are complete (figure 10).

4. Discussion

Of critical importance to the adaptation of *Artemia* to its various unforgiving habitats has been the evolution of two distinctly different paths of development (figure 3). Although the mechanisms controlling the specific path taken are poorly understood (see Clegg and Trotman 2002; Browne *et al* 1991) the importance of the production of diapause embryos (figure 2) is clear, at least for *A. franciscana* and many, if not all other bisexual species and parthenogenetic populations. Another feature of these two paths (figure 3) concerns major metabolic differences exhibited by the embryos. Thus, directly–developing embryos do not

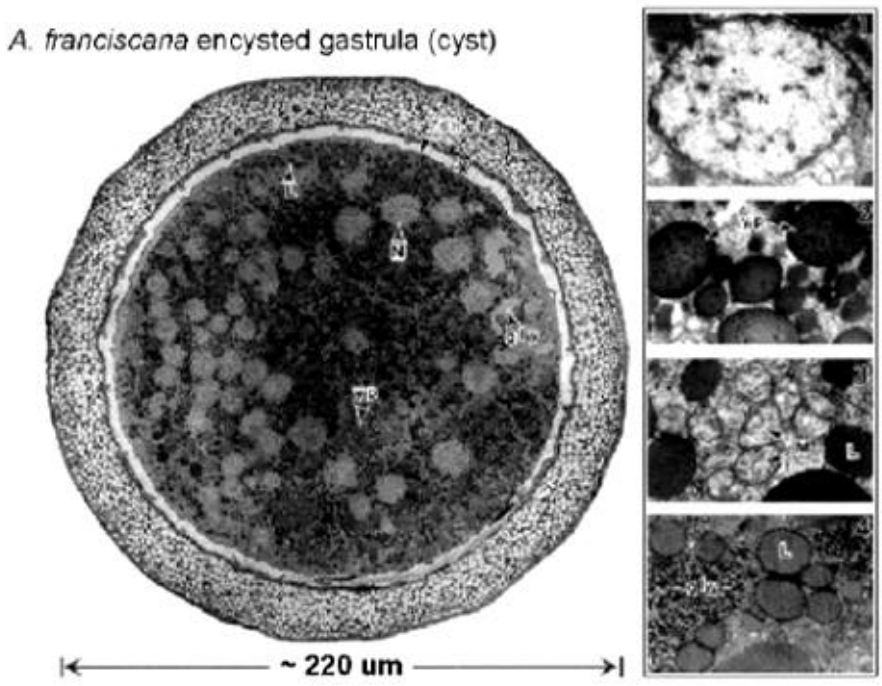

Figure 2. Cross section through an encysted embryo, and selected organelles (right panel). N, nuclei; L, lipid droplets; yp, yolk platelets; gly, glycogen rosettes; M, mitochondria. The asterisk indicates the embryonic cuticle of the shell which is not removed by decapsulation.

synthesize p26, artemin or trehalose, a disaccharide critical for desiccation tolerance (reviewed by Clegg and Trotman 2002). We interpret this to reflect a greatly reduced need for these compounds since this pathway results in a swimming nauplius which does not encounter the barrage of stresses the encysted embryo endures. That is in striking contrast to the critical role played by all three in the diapause embryo. Thus, the Hsps p26 and artemin are not induced in the usual sense of a stress response, but are programmed into a specific stage of development (figure 4). We have shown previously that trehalose synthesis follows a time course similar to these proteins (Clegg *et al* 1999). We suggest that these synthetic patterns are a result of the evolution of *Artemia*, during which the diapause embryos became programmed to anticipate stresses they will encounter soon after release from females, or even decades later. On the other hand, the availability of the direct pathway enables rapid reproduction when conditions are conducive to production and release of nauplius larvae. There is little doubt that these developmental alternatives are central to the ecological success of this primitive but widespread and highly successful crustacean.

A great deal of previous work has documented the importance of p26 as a molecular chaperone for proteins (see Liang *et al* 1997a,b; Abatzopoulos *et al* 2002; Clegg and Trotman 2002; Crack *et al* 2002; Willsie and Clegg 2002). Figure 11 is a diagrammatic representation of the overall location and function of p26 as we understand the situation today. In the absence of stress, all of the p26 is in the cytoplasm, presumably freely diffusing and not associated with cytoplasmic elements. Upon encountering stresses such as high temperature or anoxia (or when the diapause embryo forms) about half of the p26 is translocated to nuclei and probably other sites such as mitochondria and possibly the endoplasmic reticulum. These translocations are apparently initiated by a stress-induced acidification within the cells of the embryo. Translocated p26 is believed to function as a molecular chaperone for proteins while the embryo is under stress and during recovery. For example, there is abundant evidence that p26 chaperones lamins, major nuclear proteins, perhaps in association with

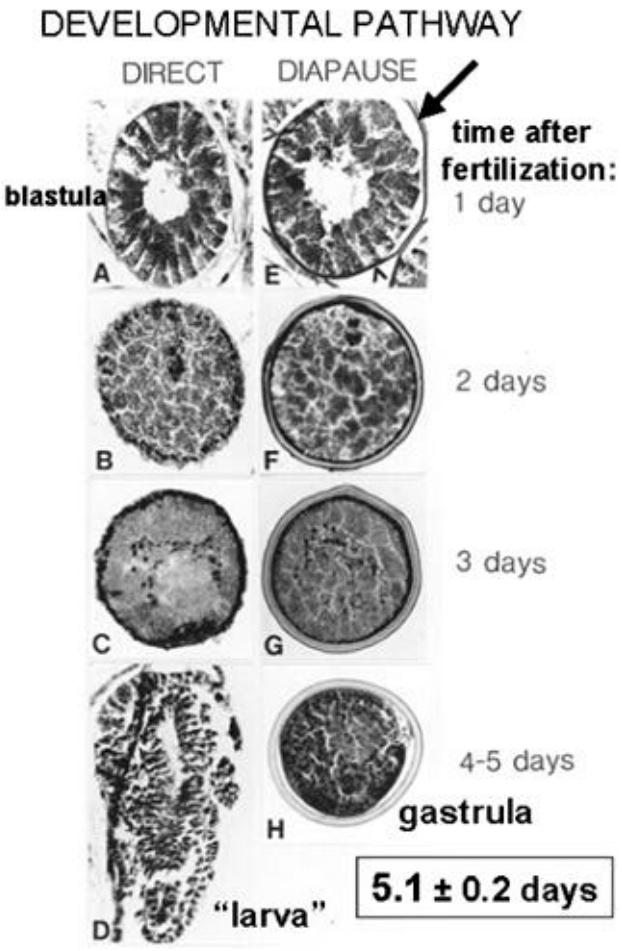

Figure 3. The two developmental pathways possible in *Artemia*. The arrow points to the embryonic cuticle present in embryos undergoing the diapause pathway, but not in those undergoing direct development. Both pathways take place in the ovisac, and require the same amount of time (mean ± SE for $n = 10$). Quotation marks around larva indicate that the larva is not yet fully complete. Modified from Jackson and Clegg (1996).

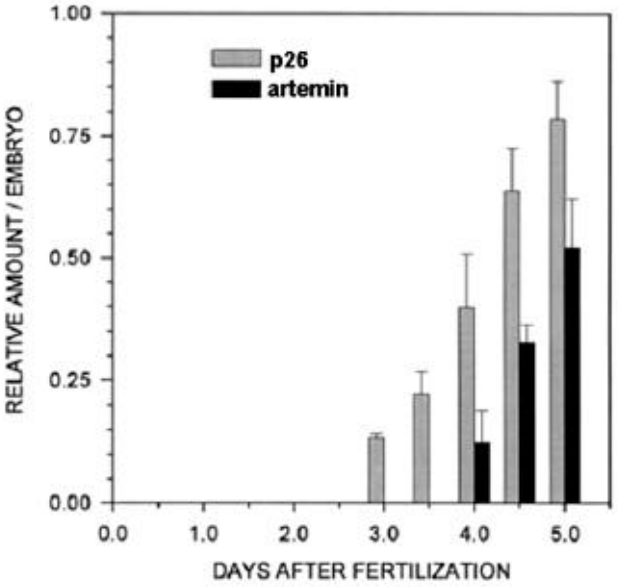

Figure 4. Appearance of p26 and artemin during development of encysted embryos (see figure 3). Vertical lines are 1 SE from the mean ($n = 10$). Data for artemin are from the present study, while those for p26 are in modified form from Jackson and Clegg (1996).

hsp70 (Willsie and Clegg 2002). All these events are reversed upon removal of the stress(es), once again being mediated by intracellular pH which, in this case, increases.

We add to this general description (figure 11) by showing here that p26 translocated to nuclei has a defined time course for exiting them following heat shock (figures 5, 6). In addition, we showed that heat shock results in an increase in aggregated protein (void volume in figure 7) also being accompanied by p26 (compare the Westerns in figure 7a, b). The picture we propose from the results in figures 5–7 is that heat shock leads to protein aggregation, p26 binds to the aggregates and either chaperones them to functional form or marks them for degradation.

We also know a great deal about the effects of anoxia on these embryos, and the behaviour of p26 which is much like that of heat shock (Clegg 1997). Figure 8 reveals that even after a year of continuous anoxia these embryos, fully hydrated and at room temperature, exhibit little indication of protein aggregation. Indeed, comparing the protein profiles of 1 week and 1 year of anoxia (figure 8), one is hard-pressed to conclude that they are appreciably different. In view of the inherent instability of globular proteins (Somero 1995) and the absence of detectable protein turnover in the cysts (Clegg 1997) one can suppose that at least some globular proteins are, indeed, unfolding but that this does not lead to aggregation because of the chaperoning activity of p26 and, perhaps, hsp70.

We have recently turned our attention to the function of artemin, a protein present in large amounts in encysted embryos (figure 5) as first shown by De Herdt *et al* (1979) and later by De Graaf *et al* (1990). Indeed, the levels and molecular masses of artemin are very similar to those of p26 (figure 5). Interestingly, both proteins appear and disappear at about the same time in development (Clegg *et al* 1994, 1995; Jackson and Clegg 1996; Liang and MacRae 1999). The amino acid sequence of artemin (De Graaf *et al* 1990) bears distinct similarity to the iron-binding protein, ferritin, although cysts also contain their own ferritin (Chen *et al* 2003). Recent studies (Warner *et al* 2004) have demonstrated that artemin purified from *A. franciscana* is exceptionally stable at high temperatures, *in vitro* and *in vivo*, and it will be interesting to study the thermal stability of artemin from other *Artemia* species, such as

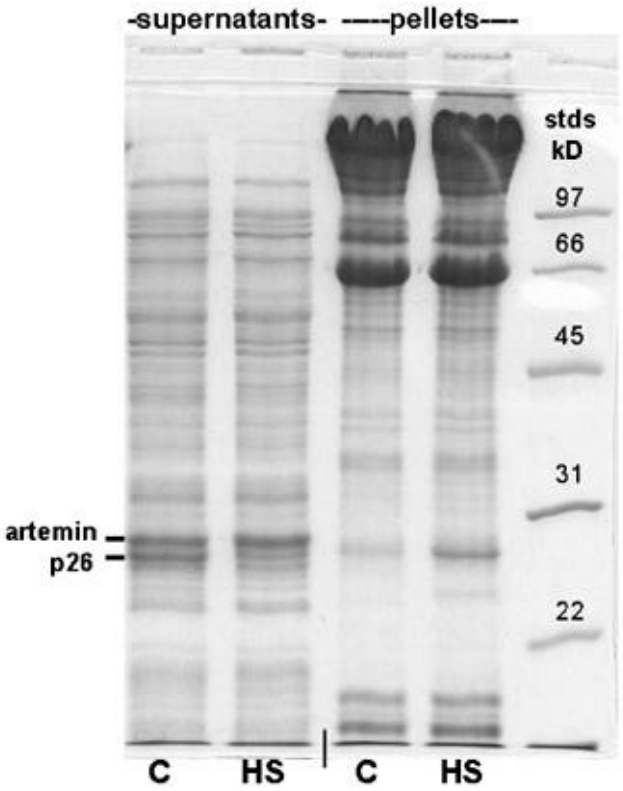

Figure 5. Analysis of artemin and p26 in control embryos (C) and those after heat shock (HS): 22 to 50°C at 0·7°/min, then incubated for another 15 min at 50°C. C and HS embryos were then homogenized (see § 2) and the low speed pellets and supernatants (1600 g, 5 min) analysed by SDS-PAGE and Coomassie staining for artemin and p26. Extensive previous work, cited in the text, has shown that all of the p26 in the pellet is contained within nuclei.

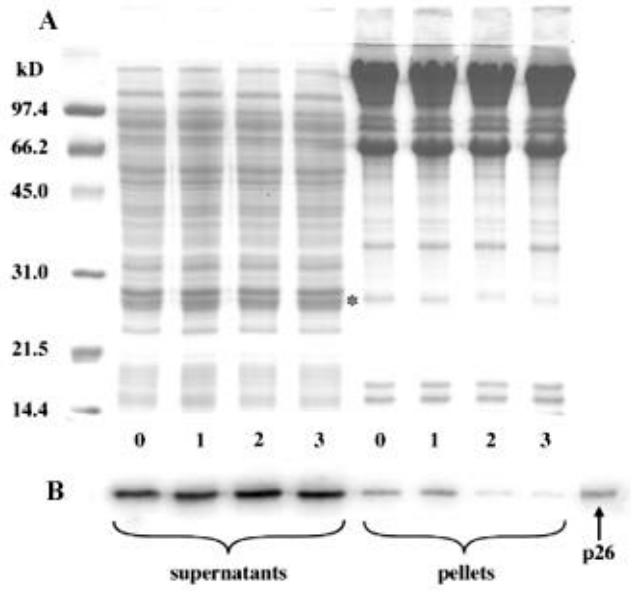

Figure 6. Results from an experiment similar to that shown in figure 5 except that the embryos were brought to 50°C, then incubated at 22°C. The numbers between **A** (Coomassie-stained gel) and **B** (Western immunoblot) refer to the incubation times after the embryos were returned to 22°C and then analysed. Thus, for '0' the embryos were homogenized immediately after heat shock, for '1' after 45 min, for '2' after 90 min and for '3' after 180 min, all at 22°C. The positive control, to the right of **B**, is 0·42 μg of pure p26.

A. tibetiana which inhabits a much colder environment. We also extended earlier work (De Herdt *et al* 1979) indicating that artemin is an RNA-binding protein. Thus, Warner *et al* (2004) obtained evidence, albeit indirect, that artemin might be an RNA chaperone (Lorsch 2002; Henics 2003). Should this possibility prove to be correct, it will mean that the encysted embryos of *Artemia* have acquired dual protection of macromolecules: p26 to protect proteins, and artemin to do the same for RNA. A unique feature of both of these proteins is that they are synthesized as part of the developmental program of encysted embryos (figure 4; Clegg and Trotman 2002) and not in response to stress. It is as if their synthesis pre-meditates stresses that these robust embryos may encounter soon, or decades after they are produced and released into the environment. This scenario is yet another example of the remarkable features of the encysted embryos of *Artemia*.

The results of the Vietnam experiment and studies of *A. tibetiana* (table 1) collectively indicate a correlation between the environmental temperature of *Artemia* and the levels of the stress proteins p26, artemin and hsp70 in the embryos of these populations (also, Clegg *et al* 2001). The ability of *A. franciscana* (SFB) to adapt to the much higher environmental temperatures in southern Vietnam has been well-documented (see Hoa 2002; Clegg *et al* 2000, 2001). Those abilities were revealed through heat-shock

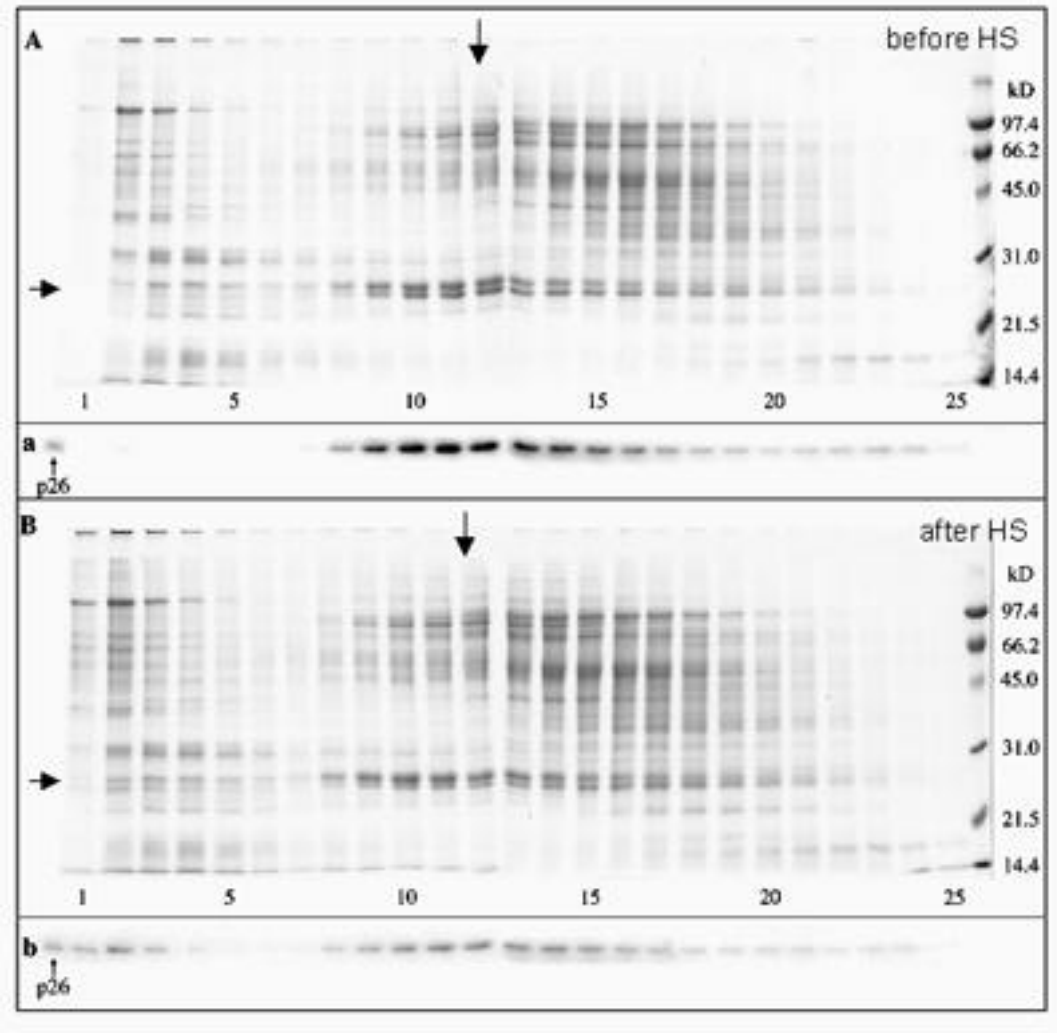

Figure 7. Coomassie-stained gels of low speed supernatants (1600 g, 5 min) from embryos before and after heat shock done the same as for '0' in figure 6. The supernatants were fractionated on Sepharose CL-6B and the resulting fractions were analysed by SDS-PAGE (**A** and **B**) and by Western blotting (**a** and **b**). The vertical arrows indicate molecular mass of about 500 kDa, approximately that of both artemin and p26. The horizontal arrows indicate about 26 kDa, the mass of the sub-unit of p26. The void volume of the column, determined by dextran blue, is represented by fractions 1 through 3. The positive control is for p26, shown to the left in **a** and **b**.

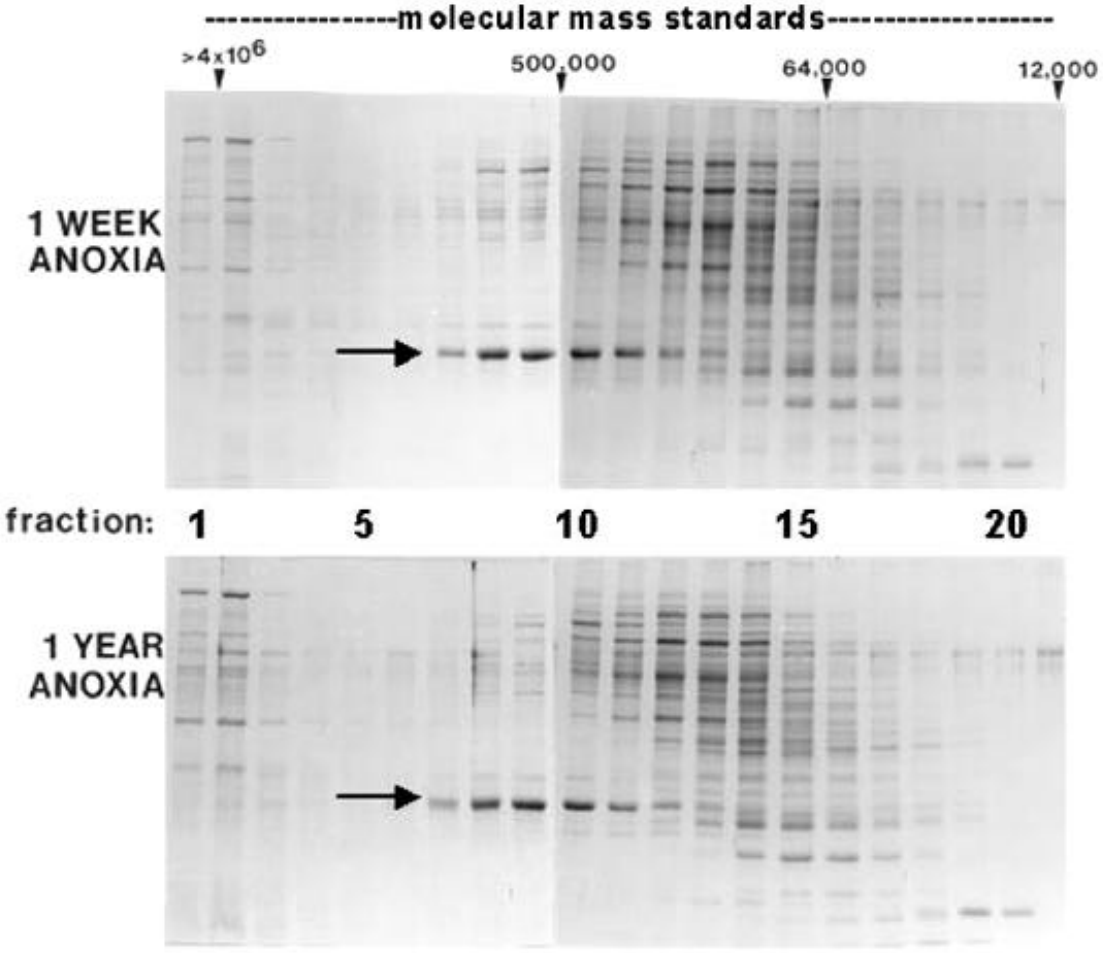

Figure 8. The profiles of proteins in low speed supernatants, analysed as in **A** and **B** of figure 7, from embryos that had experienced previous bouts of anoxia. The arrows indicate the location of artemin, not p26 which has undergone extensive anoxia-induced translocations. Fractions 1 through 3 contain the void volume of the Sepharose CL-6B gel filtration column.

Table 1. Comparison of the relative amounts of stress (heat shock) proteins in *Artemia* embryos from the Vietnam experiment and the high plateau of Tibet.

	Relative amount as a percentage of the VN-Y1 cyst content			
Embryo origin	p26	Artemin	Hsp-70_{high}	Hsp-70_{low}
VN-Y1	100	100	100	100
ISA/SFB	65	86	123	49
A. tibetiana	10	21	39	50

ISA/SFB are *A. franciscana* cysts used for the initial inoculation in the Vietnam experiment. VN-Y1 results are from cysts produced in those ponds during the first growing season, and the other data are normalized to these. The two isoforms of Hsp70 represent the high and low molecular masses of this family of proteins. Each result represents the same wet weight of all cyst samples, analysed by Western immunoblotting and densitometry.

studies in the laboratory showing that the cysts produced in the Vietnamese ponds were clearly superior to those of the inoculum from the SFB when both were exposed to temperatures of 50°C for increasing periods (Clegg *et al* 2001). In the present study we found that the increase in thermal stability was mirrored by increased levels of several stress proteins in these cysts, compared to the same proteins in the SFB inoculum (table 1). Can we safely conclude that the levels of these stress proteins and the degree of thermotolerance exhibited by the embryos produced in these very different environments are causally connected? That could be, but to paraphrase H L Mencken: 'For every

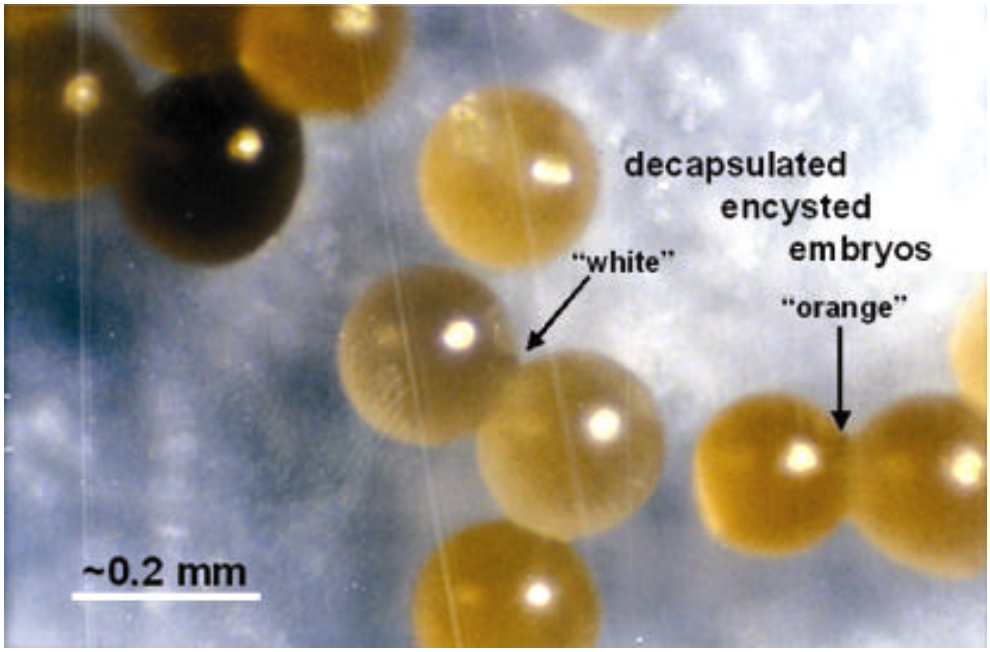

Figure 9. Several embryos whose outer shell has been removed by the decapsulation procedure (see § 2).

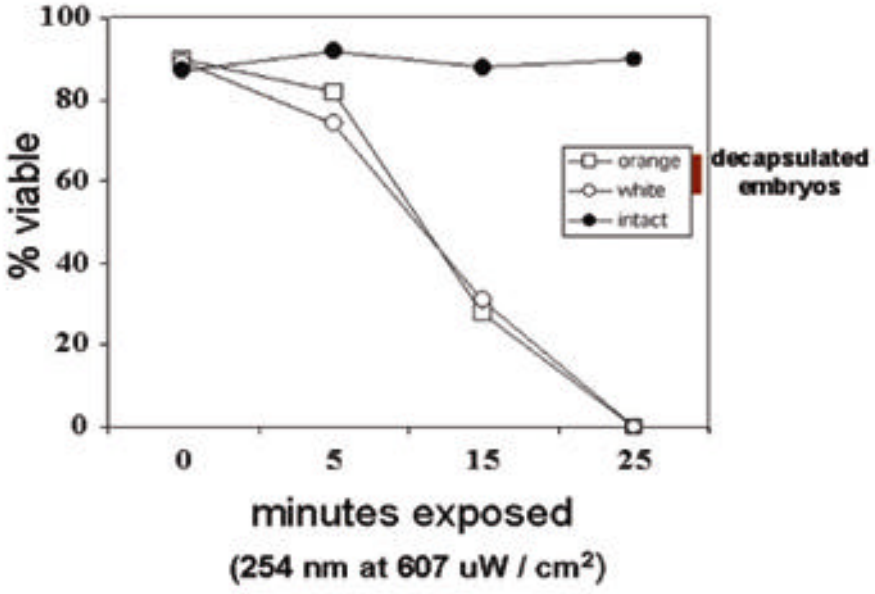

Figure 10. The effects of ultraviolet light on the viability of decapsulated embryos, and those with intact shells. The terms orange and white refer to embryos that exhibit these colours in figure 9.

complex question there is an easy, simple answer, and it's wrong'. Thus, we think it is likely that factors in addition to stress proteins could be involved in the adaptation of animals from the SFB growing in the much hotter ponds in Vietnam.

We suggest that *A. tibetiana* has reduced the levels of these stress proteins since, at the very low temperature of their environment, there is no need to maintain them at the high levels seen in the Vietnamese-grown cysts, for example. That interpretation is in keeping with the rather obvious idea that there is a cost attached to maintaining high levels of these proteins, and that it is advantageous to escape those costs whenever possible, something that *A. tibetiana* seems to have done to a marked degree, at least in the case of p26 and artemin.

We believe that one of the most interesting outcomes of the research reported here concerns the importance of the outer part of the embryo's shell in protection against ultraviolet irradiation (figures 9 and 10). We initially assumed that the embryonic cells would contain large amounts of anti-oxidant compounds and enzymes to protect against the effects of UV irradiation which these embryos encounter in nature. Thus, *Artemia* embryos spend long periods of time, sometimes years or possibly even decades, floating on the surface of hypersaline bodies of water, or being washed on to shore, where they are bombarded by UV in both cases. Yet, what little we know about anti-oxidants, and other UV-protectants, indicates that the embryos do not contain an arsenal of these compounds, with the possible exception of carotenoids. Algae provide a major food source for *Artemia*, and these autotrophs contain abundant concentrations of carotenoids, particularly when growing under hypersaline conditions. These fat-soluble compounds are incorporated into the oocytes of *Artemia* and, therefore, can become a major component of the lipids of encysted embryos. However, that is highly variable as indicated by the different degrees of 'orangeness' of the decapsulated embryos (figure 9). Carotenoids, in general, are considered to be very good antioxidants, playing important roles in the detoxification processes that neutralize free radicals. The literature on these compounds is large, but Nelis *et al* (1989) have written the definitive review on carotenoids in life cycle stages of *Artemia* – their review is highly recommended.

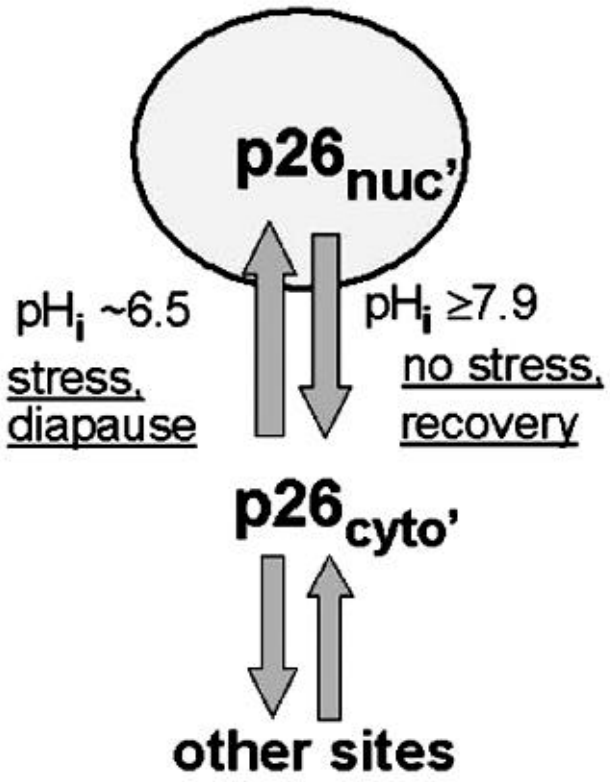

Figure 11. Diagrammatic representation of what we believe represents the overall intracellular locations and functions of intracellular p26, where nuc' is nucleus and cyto' is cytoplasm.

Surprisingly little work has been done on other anti-oxidant systems in encysted *Artemia* embryos (reviewed by Clegg and Trotman 2002). Based on current results it seems that encysted embryos do not contain large concentrations of molecules that protect against and/or repair damage from UV. In our opinion the best work has been done by Rudneva (1999) who used cysts collected from Sakskoe Lake (Crimea, Ukraine), probably also a parthenogenetic population (Triantaphyllidis *et al* 1998) in a detailed and comparative study. She found that cysts contained similar or even lower levels of vitamins A and C, and glutathione compared to eggs of mussels (*Mytilus galloprovincialis*) and to eggs and larval stages of three fish species. In addition, these cysts did not contain elevated levels of lipoxygenase, superoxide dismutase, catalase, peroxidase or glutathione reductase activities compared to eggs and larvae of the other species. To explain these interesting results, Rudneva (1999) suggested that the thick shell of *Artemia* cysts might protect the embryo from oxidative damage. It is not clear to us whether the basis for this suggestion involves the screening and/or reflection of UV radiation by the shell, or prevention of the passage of molecular oxygen across it. Our results (figures 9 and 10) certainly are consistent with the former possibility put forth by Rudneva (1999) and indicate just that – the presence of a shell that seems to essentially be a 'sunscreen' (figures 9 and 10). We view this as an ingenious solution – structural protection that does not require any cellular metabolic investment and renewal. We note that a similar strategy sems to have been aquired by certain tunicates where extra-embryonic cells play the role of a 'sunscreen' (Epel *et al* 1999). The latter paper also provides an excellent account of the literature that is relevant to our work on *Artemia*.

Finally, we note an interesting possibility, suggested by a reviewer of this paper, that phenolic tanning and/or melanization of the outer cyst shell might be involved in the UV resistance of encysted embryos. These processes are general features of the arthropod cuticle, including embryonic envelopes (Elsherief 1993). To our knowledge nothing is known about this in the case of *Artemia* cysts, so this possibility remains to be examined.

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References

Abatzopoulos T, Zheng B and Sorgeloos P 1998 *Artemia tibetiana*: preliminary characterization of a new *Artemia* species found in Tibet (People's Republic of China). International Study on *Artemia* LIX; *Int. J. Salt Lake Res*. **7** 41–44

Abatzopoulos T J, Beardmore J A, Clegg J S and Sorgeloos P (eds) 2002 *Artemia*: *Basic and applied biology* (Dordrecht: Kluwer Academic Publishers) pp 1–286

Arrigo A-P and Müller W E G (eds) 2002 *Small stress proteins* (Berlin: Springer-Verlag) pp 1–270

Baert P, Anh N T, Quynh V D, Hoa N V and Sorgeloos P 1997 Increasing cyst yields in *Artemia* culture ponds in Vietnam: the multi-cycle system; *Aquaculture Res*. **28** 809–814

Beissinger M and Buchner J 1998 How chaperones fold proteins; *Biol. Chem*. **379** 245–259

Browne R A and Wanigasekera G 2000 Combined effects of salinity and temperature on survival and reproduction of five species of *Artemia*; *J. Exp. Mar. Biol. Ecol*. **244** 29–44

Browne R A, Sorgeloos P and Trotman C N A (eds) 1991 *Artemia biology* (Boca Raton: CRC Press) pp 1–374

Chen T, Amons R, Clegg J S, Warner A H and MacRae T H 2003 Molecular characterization of artemin and ferritin from *Artemia franciscana*; *Eur. J. Biochem*. **270** 137–145

Clegg J S 1997 Embryos of *Artemia franciscana* survive four years of continuous anoxia: the case for complete metabolic rate depression; *J. Exp. Biol*. **200** 467–475

Clegg J S and Jackson S A 1998 The metabolic status of quiescent and diapause embryos of *Artemia franciscana*; *Arch. Hydrobiol*. **52** 425–439

Clegg J S and Trotman C N A 2002 Physiological and biochemical aspects of *Artemia* ecology; in *Artemia*: *Basic and applied biology* (eds) Th J Abatzopoulos, J A Beardmore, J S Clegg and P Sorgeloos (Dordrecht: Kluwer Academic Publishers) pp 129–170

Clegg J S, Jackson S A and Warner A H 1994 Extensive intracellular translocations of a major protein accompany anoxia in embryos of *Artemia franciscana*; *Exp. Cell Res*. **212** 77–83

Clegg J S, Jackson S A, Liang P and MacRae T H 1995 Nuclear-cytoplasmic translocations of protein p26 during aerobic-anoxic transitions in embryos of *Artemia franciscana*; *Exp. Cell Res*. **219** 1–7

Clegg J S, Willsie J K and Jackson S A 1999 Adaptive significance of a small heat shock/α-crystallin protein in encysted embryos of the brine shrimp, *Artemia franciscana*; *Am. Zool*. **39** 836–847

Clegg J S, Jackson S A, Hoa N V and Sorgeloos P 2000 Thermal resistance, developmental rate and heat shock proteins in *Artemia franciscana*, from San Francisco Bay and southern Vietnam; *J. Exp. Mar. Biol. Ecol*. **252** 85–96

Clegg J S, Hoa N V and Sorgeloos P 2001 Thermal tolerance and heat shock proteins in encysted embryos of *Artemia* from widely different thermal habitats; *Hydrobiologia* **466** 221–229

Coleman J S, Heckathorn S A and Hallberg R L 1995 Heat shock proteins and thermotolerance: linking molecular and ecological perspectives; *Trends Ecol. Evol*. **10** 305–306

Crack, J A, Mansour M, Sun Y and MacRae T H 2002 Functional analysis of a small heat shock/alpha-crystallin protein from *Artemia franciscana*. Oligomerization and thermotolerance; *Eur. J. Biochem*. **269** 1–10

Decleir W, Moens L, Slegers H, Sorgeloos P and Jaspers E 1987 *Artemia research and its applications* vol. 2 (Wetteren: Universa Press) pp 1–541

De Graaf J, Amons R and Möller W 1990 The primary structure of artemin from *Artemia* cysts; *Eur. J. Biochem*. **193** 737–750

De Herdt E, Slegers H and Kondo M 1979 Identification and characterization of a 19-S complex containing a 27,000-Mr protein in *Artemia salina*; *Eur. J. Biochem*. **96** 423–430

Ellis R J 1999 Molecular chaperones: pathways and networks; *Curr. Biol*. **9** R137–R139

Ellis R J and Hartl F U 1999 Principles of protein folding in the cellular environment; *Curr. Opin. Struct. Biol*. **9** 102–110

Elsherief S S 1993 Histochemical characteristics of the egg membranes of *Portunus pelagicus* (L); *Acta Biol. Hungarica* **44** 269–280

Epel D, Hemela K, Shick M and Patton C 1999 Development in the floating world: defenses of eggs and embryos against damage from UV radiation; *Am. Zool*. **39** 271–279

Feder M E and Block B A 1991 On the future of animal physiological ecology; *Funct. Ecol*. **5** 136–144

Feder M E and Hofmann G E 1999 Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology; *Ann. Rev. Physiol*. **61** 243–282

Fiege U, Morimoto R I, Yahara I and Polla B S (eds) 1996 *Stress-inducible cellular responses* (Berlin: Birkhäuser-Verlag) pp 1–492

Fink A L 1999 Chaperone-mediated protein folding; *Physiol. Rev*. **79** 425–449

Frankenberg M M, Jackson S A and Clegg J S 2000 The heat shock response of adult *Artemia franciscana*; *J. Thermal Biol*. **25** 481–490

Henics T 2003 Extending the "stressy" edge: molecular chaperones flirting with RNA; *Cell Biol. Int*. **27** 1–6

Hightower L E 1998 The promise of molecular biomarkers for environmental Monitoring; *Biol. Chem*. **379** 1213–1215

Hoa N V 2002 *Seasonal farming of the brine shrimp Artemia francescana in artisanal salt ponds in Vietnam: effects of temperature and salinity*, Ph.D. thesis, Applied Biological Sciences, Ghent University, Gent, Belgium

Jackson S A and Clegg J S 1996 The ontogeny of low molecular weight stress protein p26 during early development of the brine shrimp, *Artemia franciscana*; *Dev. Growth Differ*. **38** 153–160

Krebs R A 1999 A comparison of Hsp 70 expression and thermotolerance in adults and larvae of three *Drosophila* species; *Cell Stress Chaperones* **4** 243–249

Laemmli U K 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4; *Nature* (*London*) **227** 680–685

Liang P and MacRae T H 1999 The synthesis of a small heat shock/*α*-crystallin protein in *Artemia* and its relationship to stress tolerance during development; *Dev. Biol*. **207** 445–456

Liang P, Amons R, MacRae T H and Clegg J S 1997a Purification, structure and molecular chaperone activity in vitro of *Artemia* p26, a small heat shock/*α*-crystallin protein; *Eur. J. Biochem*. **243** 225–232

Liang P, Amons R, Clegg J S and MacRae T H 1997b Molecular characterization of a small heat-shock/*α*-crystallin protein from encysted *Artemia* embryos; *J. Biol. Chem*. **272** 19051–19058

Lorimer G H and Baldwin T O 1998 Molecular chaperones; *Methods Enzymol*. **290** 1–500

Lorsch J R 2002 RNA chaperones exist and DEAD box proteins get a life; *Cell* **109** 797–800

MacRae T H, Bagshaw J C and Warner A H (eds) 1989 *Biochemistry and cell biology of Artemia* (Boca Raton: CRC Press) pp 1–264

Morimoto R I, Tissières A and Georgopoulos C (eds) 1994 *The biology of heat shock proteins and molecular chaperones* (New York: Cold Spring Harbor Laboratory Press) pp 1–610

Nelis H J, Lavens P, Moens L and Sorgeloos P 1989 Carotenoids in relationship to *Artemia* development; in *Biochemistry and cell biology of Artemia* (eds) T H MacRae, J C Bagshaw and A H Warner (Boca Raton: CRC Press) pp 159–190

Norris C E, Dilorio P J, Schultz R J and Hightower L E 1995 Variation in heat shock proteins within tropical and desert species of Poeciliid fishes; *Mol. Biol. Evol*. **12** 1048–1062

Persoone G, Sorgeloos P, Roels O and Jaspers E (eds) 1980 *The brine shrimp, Artemia* vol. 2 (Wetteren: Universa Press) pp 1–636

Richardson A, Landry S J and Georgopoulos C 1998 The ins and outs of a molecular chaperone machine; *Trends Biochem. Sci*. **23** 138–143

Rudneva I I 1999 Antioxidant systems of Black Sea animals in early development; *Comp. Biochem. Physiol. C* **122** 265–271

Somero G N 1995 Proteins and temperature; *Ann. Rev. Physiol*. **57** 43–68

Tomanek L and Somero G N 1999 Evolutionary and acclimation-induced variation in the heat–shock responses of congeneric marine snails (Genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *J. Exp. Biol*. **202** 2925–2936

Triantaphyllidis G V, Abatzopoulos T J and Sorgeloos P 1998 Review of the biogeography of the genus *Artemia* (Crustacea, Anostraca); *J. Biogeogr*. **25** 213–226

van den Ijssel P, Norman D G and Quinlan R A 1999 Molecular chaperones: small heat shock proteins in the limelight; *Curr. Biol*. **9** R103–R105

Van Stappen G 2002 Zoogeography; in *Artemia: Basic and applied biology* (eds) T H Abatzopoulos, J A Beardmore, J S Clegg and P Sorgeloos (Dordrecht: Kluwer Academic Publishers) pp 171–224

Van Stappen G, Sui L, Xin N and Sorgeloos P 2003 Characterization of high-altitude *Artemia* populations from the Qinghai-Tibet plateau, PR China; *Hydrobiologia* **500** 179–192

Warner A H, MacRae T H and Bagshaw J C (eds) 1989 *Cell and molecular biology of artemia development* (New York: Plenum Press) pp 1–453

Warner A H, Brunet R T, MacRae T H and Clegg J S 2004 Artemin is an RNA-binding protein with high thermal stability and potential RNA chaperone activity; *Arch. Biochem. Biophys.* **424** 189–200

Willsie J K and Clegg J S 2002 Small heat shock protein p26 associates with nuclear lamins and HSP70 in nuclei and nuclear matrix fractions from stressed cells; *J. Cell. Biochem.* **84** 601–614

Zunying L, Mianping Z and Jian L 1998 A study of *Artemia* in Lagkor Co, Tibet: Biological features; *J. Lake Res*. **10** 92–96 (in Chinese)

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