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Thermal resistance, developmental rate and heat shock proteins in *Artemia franciscana,* from San Francisco Bay and southern Vietnam

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Abstract

Cysts (encysted gastrula embryos) of *Artemia franciscana* collected from salterns in San Francisco Bay, California, USA (SF) were inoculated into much warmer growth ponds in the Mekong Delta region of Vietnam (V) in 1996. V adults arising directly from these cysts during 17 April to 15 May produced their own cysts, which were collected, processed and stored until shipped to the USA for study. Adults grown in the laboratory from SF cysts (those used for the inoculation) were less resistant to high temperature than adults cultured from V cysts. V cysts produced heat-resistant adults, even though cultured under the same laboratory conditions as SF animals, at much lower temperatures than they ever experienced in Vietnam. Differences in thermal performance between SF and V adults were retained in the second generation, cultured from cysts produced in the laboratory by first generation adults, suggesting a genetic basis for the better heat resistance of V adults. We propose that the operation of natural selection in the Vietnam growth ponds produced adults with improved thermal tolerance, and that the basis for this tolerance was incorporated into the developmental program of their cysts. Surprisingly, differences in heat resistance of laboratory reared animals were not reflected in constitutive levels of the hsp70 family which were similar in first generation SF and V adults. A conditioning heat shock (HS, 37°C, 30 min) led to the same level of induced thermotolerance in SF and V first generation adults when evaluated 24 h post-HS. Levels of hsp70 were also up-regulated at that time, but to about the same extent in SF and V adults. Developmental rates of SF cysts used for the inoculation were faster than those of cysts produced in Vietnam when both were incubated at 21±1°C, suggesting that V cysts have become adapted to develop at higher temperatures.

Keywords: *Artemia*; Heat resistance; Induced thermotolerance; Heat shock; Hsp70; P26; Artemin

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1. Introduction

Until recently, research on the stress (heat shock) response and associated stress proteins and molecular chaperones focused chiefly on cellular and molecular aspects (for example, Morimoto et al., 1994; Fiege et al., 1996; Beissinger and Buchner, 1998; Fink and Golo, 1998; Lorimer and Baldwin, 1998; Richardson et al., 1998; Ellis, 1999, 2000; Ellis and Hartl, 1999; Fink, 1999; van den Ijssel et al., 1999). As pointed out in the review by Feder and Hofmann (1999) the focus is changing as more attention is given to the evolutionary and ecological aspects of this important and ubiquitous response (also see Coleman et al., 1995; Feder, 1999; Krebs, 1999; Tomanek and Somero, 1999). The comparative approach has several advantages, one of which involves the choice of organisms that provide special opportunities for study because of their particular ecological setting. Such an organism is *Artemia*, a primitive crustacean known as the brine shrimp. Species of *Artemia* are found in a wide variety of harsh environments world wide (Triantaphyllidis et al., 1998) and exhibit an impressive repertoire of adaptations enabling them to cope with these challenging conditions (Clegg and Conte, 1980; Persoone et al., 1980; Decleir et al., 1987; MacRae et al., 1989; Warner et al., 1989; Browne et al., 1991; Hand and Hardewig, 1996). Of major importance in *Artemia*'s life history is the production of encysted dormant embryos (cysts) that can survive severe desiccation, repeated bouts of desiccation/rehydration, prolonged anoxia when fully hydrated, temperature extremes and exposure to high doses of ultraviolet and other forms of radiation (references just cited and Clegg, 1997; Clegg et al., 1999). These gastrula embryos ($\sim$4000 nuclei) are arguably the most resistant of all animal life history stages to extremes of environmental stress. While the response of cysts to stressful conditions has been examined in some detail, little attention has been given to other life cycle stages, and that is one motive for the present work which focuses on adults.

In 1996 a research project was initiated (the Vietnam Experiment) in which cysts from San Francisco Bay (SF) were inoculated into controlled, experimental growth ponds in the Mekong Delta region of Vietnam (Baert et al., 1997). Cysts produced in Vietnam (V) that year were used for inoculation in the second year (1997) and so on, to the present time. We know that cysts produced in these ponds are substantially more resistant to high temperatures when compared to those used for the inoculation and, importantly, that this superior performance was achieved during the first growing season following inoculation (Clegg et al., 2000). We were interested in determining whether adults raised in the laboratory from these Vietnam-grown cysts would also have improved thermal tolerance compared to adults grown from SF cysts. Previous work suggested that they would (Frankenberg et al., 2000) although animals from poorly controlled ponds were used, and other uncertainties in that study prevented an unambiguous conclusion.

We will show that adults produced in the laboratory from V cysts do indeed exhibit greater resistance to high temperatures compared to those cultured from SF cysts and, importantly, that this difference is retained in the second generation of laboratory-grown SF and V adults. We also found that differences in the heat resistance of intact adults were not reflected in any obvious way to relative levels of the hsp70 family, although a

conditioning heat shock did lead to the induction of thermotolerance and an up-regulation of hsp70 in both SF and V adults.

2. Materials and methods

2.1. Origins of cysts and hatching assays

A. franciscana cysts from San Francisco Bay (SF) USA were obtained from the *Artemia* Reference Center, Ghent University, Gent, Belgium, as part of the cyst collection used for the International Study of *Artemia* (ISA). These cysts (ISA-1258) were inoculated into culture ponds in Vietnam on 17 April 1996 and, after hatching, gave rise to adults, the first appearing about 10 days later. These adults produced their own cysts which were harvested daily during the remaining culture period which ended on 15 May; thus, only the first crop of adults arising from the inoculation was involved in cyst production. This part of the Vietnam Experiment 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 region (see Baert et al., 1997). Pond salinity was 78.3±3.1 ppt and water temperatures ranged from a low of 24°C (early morning) to a high of 38°C (mid-afternoon) during the growth period. The average daily temperature at 2 p.m. was 35.3±0.6°C. After processing, samples of these cysts were shipped to Ghent University, Belgium, and then to the USA where the results in this paper were obtained.

Cyst viability and developmental rate were measured using 20-well plastic depression plates, each well containing 10–30 cysts in 400 μl of seawater (SW). The plates were covered, sealed with tape to prevent evaporation, and incubated in constant light at 21±1°C. The percentage of embryos hatching into nauplius larvae was plotted as a function of incubation time, and the time needed to produce one-half the final hatching level was obtained. This value ($t_{1/2}N$) is a reliable measure of developmental rate (Clegg, 1992).

2.2. Adult culture, heat shock (HS) and protein preparation

Adults were grown in the laboratory from samples of the same SF cysts used for the inoculum (ISA-1258) and also from the V cysts produced in the Vietnam growth ponds. These cultures were carried out side-by-side using 300-ml plastic bowls filled with a monoculture of the unicellular alga, *Isochrysissp.*, in filtered SW nutrified with Guillard's F medium. Animals were also fed a C-5 algal diet as needed [45% *Thalassiosira pseudonana* (clone 3H), 35% *Skeletonema sp.*, 15% *Chaetoceros calcitrans* and 5% *Isochrysis galbana* (clone T-iso)]. Care was taken to ensure that the two groups of adults, referred to as first generation SF and V adults, were fed the same amount, at the same time. The temperature averaged 21±1°C during culture, and the time required to produce adults from hatched cysts was 2–3 weeks. These first generation SF and V adults were used for experiments within 2 weeks of reaching maturity.

Adults of the first generation (SF and V) were also allowed to produce their own cysts in the laboratory using the same culture methods. These cysts, collected over 2 weeks following adult maturation, were desiccated to terminate diapause (Drinkwater and Clegg, 1991) and then hatched into nauplius larvae, from which the 'second generation of adults' (SF and V) was obtained in laboratory cultures.

For heat exposure, adults were collected on cloth filters that were blotted on paper towels to produce a compact pile of undamaged animals. These were transferred into 50-ml plastic centrifuge tubes containing 35 ml of aerated SW preheated to the appropriate temperature. Each tube contained 12 or 15 males and 12 or 15 females, as mated pairs in all cases. Incubation was carried out using a Lauda RM waterbath with gentle aeration at 38.0±0.05°C, a temperature that is eventually lethal, and animals were removed after different exposure times. Survival was monitored over 3 days of incubation at room temperature (21±1°C) after which further mortality was very rare. To evaluate induction of thermal tolerance, a conditioning sublethal heat shock (HS) was given (37.0±0.05°C, 30 min) determined previously from LT_{50} studies (Frankenberg et al., 2000). After a 24-h recovery period, the animals were incubated at 38.0±0.05°C for 1 h and survival followed as above. Results were evaluated for significance using Student's *t*-test.

For stress protein analysis animals were collected on cloth filters and rinsed with ice-cold distilled water (~ 5 s) to remove external SW. After blotting the cloths on paper towels for 1 min, the animals were weighed and homogenized on ice at 200 mg wet wt ml^{-1} of buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4). Buffer K also contained a broad spectrum protease inhibitor cocktail (Complete™ Mini, from Boehringer Mannheim, Germany) used at its strongest recommended level (1 tablet for 7 ml buffer K). Homogenate aliquots were combined separately with equal volumes of 2×SDS sample buffer, vortexed and heated at 100°C for 5 min (Laemmli, 1970). After cooling, these preparations were centrifuged (1630 *g*, 3 min) to remove chitinous exoskeleton fragments that prevented accurate pipetting. Sample volumes of 9 μl per lane were used in all cases, representing the same wet weight of animals (0.9 mg) and enabling direct comparison between different samples.

Cysts were prepared similarly after being hydrated in SW at 2°C for 16–18 h. After collection on cloth filters, and brief washing with distilled water (10 s) the filters were blotted on paper towels for 2 min to remove inter-cyst water. Cysts were homogenized on ice at 100 mg wet wt ml^{-1} of buffer K, and aliquots combined separately with equal volumes of 2×SDS sample buffer, vortexed and heated at 100°C for 5 min (Laemmli, 1970). After cooling, the preparations were centrifuged (1630 *g*, 3 min) to remove insoluble fragments of the chitinous shells that prevent accurate pipetting. Aliquots of 8 μl of these preparations, equivalent to 0.4 mg wet weight of cysts, were applied to each lane of the gels.

2.3. SDS–PAGE and Western immunoblotting

SDS preparations were electrophoresed on 12% polyacrylamide gels. For each experiment, two identical gels were run: one was stained with Coomassie Blue [500 mg

Serva Blue 500 ml^{-1} of methanol–acetic acid–water (40:50:10, v/v)] and proteins on the other were transferred to a nitrocellulose membrane (Towbin et al., 1979) for Western immunoblot analysis. Membranes were incubated with monoclonal anti-hsp70 antibody, clone 7.10, that recognizes both constitutive and induced isoforms (Affinity BioReagents, Golden, CO, USA). Goat anti-rat IgG conjugated with horseradish peroxidase was used for the secondary antibody (Southern Biotechnology Associates, Birmingham, AL, USA). ECL Western Blotting Reagents (Amersham, Arlington Heights, IL, USA) were used for detection of labeled proteins on Kodak BioMax MR single emulsion film (Eastman Kodak, Rochester, NY, USA) and developed in an X-Ray film processor. Similar protocols were used to detect artemin, a ferritin-like stress protein present in cysts (De Herdt et al., 1979; De Graaf et al., 1990) and p26, a small heat shock/α-crystallin protein of known importance to cyst stress resistance (see Clegg et al., 1999; Liang and MacRae, 1999). Differences from the Western blotting described above for hsp70 involved the use of anti-artemin, provided by Herman Slegers, and our anti-p26, both polyclonal (see Clegg et al., 1994; Jackson and Clegg, 1996 for further details).

3. Results

3.1. Heat resistance and induced thermotolerance

First generation adults produced in the laboratory from V cysts were more tolerant to high temperatures than adults raised from the inoculated SF cysts (Fig. 1). At this eventually lethal temperature (38°C) the time required to kill 50% of the animals was a little over 60 min for V adults, and about 35 min for SF animals. Because of large errors the difference between SF and V mean survival after 45 min of exposure is not quite significant ($P = 0.09$), unlike results for 60 min which are clearly different ($P < 0.01$). No consistent differences between male and female death rates were noted for either SF or V adults.

Induction of thermotolerance was examined by exposing first generation SF and V adults to a conditioning, sublethal heat shock (HS) of 37°C for 30 min, then allowing a 24-h recovery period at room temperature (21±1°C). At that time the animals were incubated at the eventually lethal temperature of 38°C for 1 h and their survival followed for the next 3 days. The level of induced thermotolerance was impressive, with a survival level of 85% seen for both SF and V adults (a total of 30 mated pairs was examined in both cases) with nearly equal numbers of male and female survivors.

3.2. Stress proteins and protein profiles of cysts and adults

Fig. 2 shows Coomassie-stained proteins of SF and V cysts and first generation adults grown from them in the laboratory (part A) and Western immunoblots for detection of hsp70 (part B) and p26 and artemin (part C). The protein profiles of SF and V cysts are

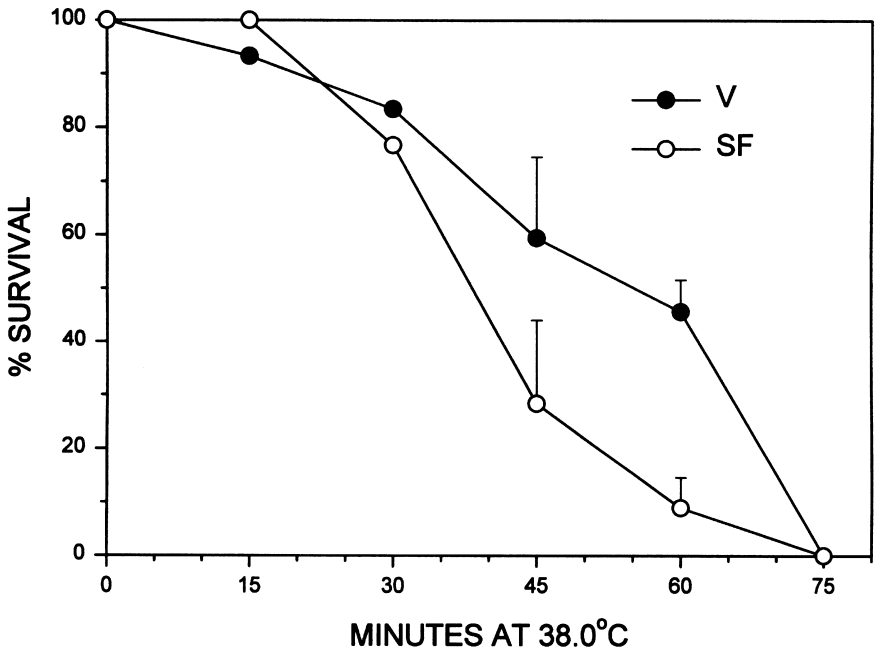

Fig. 1. Survival of first generation adults cultured in the laboratory from San Francisco Bay cysts (SF) and from cysts produced in Vietnam (V). Survival was monitored at 21±1°C for 3 days following heat exposure. Data points without error bars represent 30 animals (15 mated pairs); the others show means for 3 sets of 15 mated pairs (90 total animals)±standard errors.

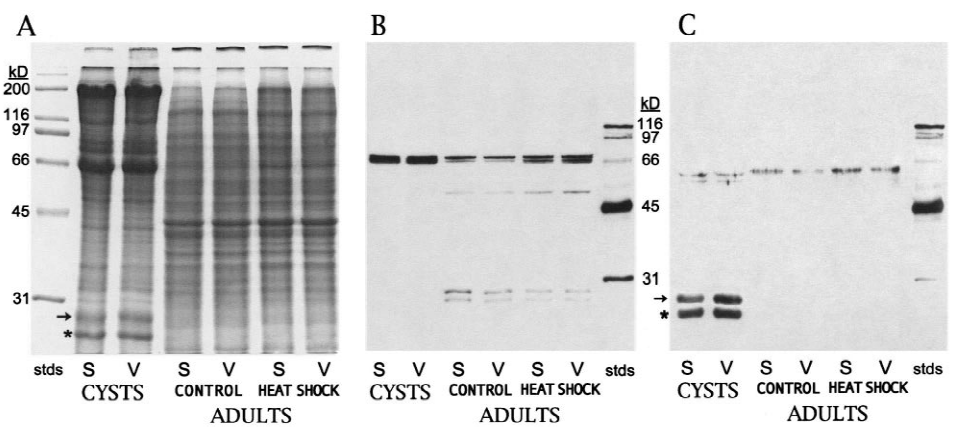

Fig. 2. Coomassie-stained proteins in San Francisco Bay cysts (S) and cysts produced in Vietnam (V), and in first generation adults cultured from them in the laboratory (part A). Parts B and C are Western immunoblots for the detection of hsp70 proteins, p26 (asterisk) and artemin (arrow). Heat shocks were for 30 min at 37°C, followed by a 24-h recovery period when the animals were prepared for electrophoresis.

very similar, as are those for adults except that there appears to be slightly more protein per unit wet weight in heat shocked adults compared to controls, and particularly so for proteins of molecular mass greater than about 150 kDa (Fig. 2A). We observed that difference frequently during the present study, and previously (Frankenberg et al., 2000). The Western blot in Fig. 2B indicates that similar levels of the two hsp70 proteins are present in SF and V cysts; adults cultured from these cysts also have comparable amounts of these proteins. Heat shocked SF and V adults up-regulated these two hsp70 proteins as indicated at 24 h after the conditioning heat shock. These increases coincided with the observed induction of thermotolerance (see previous section). No induced form of hsp70 was observed in these studies so, strictly speaking, these constitutive proteins should be termed hsc70.

Comment is required about proteins of molecular mass well below 70 kDa detected on the Western shown in Fig. 2B, something we have seen repeatedly in extracts of adults (Frankenberg et al. 2000). We cannot rule out that they originate from hsp70 proteolysis, but any protease involved would have to be active in the presence of high concentrations of a broad spectrum of eight protease inhibitors used in the homogenizing buffer (see Materials and methods). Also, there is no indication of general protein breakdown in the Coomassie-stained profiles (Fig. 2A). It seems most likely to us that the proteins in question simply have an epitope recognized by the anti-hsp70 we used, and have no significance in the context of our study.

Two major cyst proteins were also examined (Fig. 2C). This immunoblot indicates that SF and V cysts contain similar amounts of the small heat shock/α-crystallin, p26 (asterisk), and the ferritin-like protein, artemin (arrow), although the latter might be present in slightly higher amounts in V cysts. Fig. 2C shows clearly that adults of both groups do not contain these major cyst proteins in detectable amounts, a point of importance when interpreting results on the stress resistance of different life history stages of *Artemia*.

3.3. Developmental rates of SF and V cysts

We measured the rate at which these cysts hatched into swimming nauplius larvae when incubated in SW at room temperature (21–23°C). From plots of percentage hatching versus incubation time we extracted the values of $t_{1/2}N$ (time for 50% of the final hatching level to be reached). V cysts developed slower under these conditions ($t_{1/2}N \sim 33$ h) compared to SF cysts ($t_{1/2}N \sim 23$ h). Both groups of cysts had final hatching levels close to 90%, so our results are not influenced by differences between the viability of inoculated SF cysts and those harvested from the ponds in Vietnam.

3.4. Adults of the second V generation are also more heat resistant than SF

Second generation SF and V adults were subjected to 38.0±0.05°C for 1 h and their survival was followed over the next 3 days. SF adult survival was 16.7±9.4% and that of V adults was 48.6±5.5% (in both cases, $n=6$ groups of 12 mated pairs). Clearly, V

adults are more heat tolerant than SF adults, both of the second generation. Use of Student's *t*-test indicates that neither of these survival levels is significantly different from those observed for first generation adults of each group under the same conditions: 8.9±5.7% (SF) and 45.6±6.9% (V), these values being taken from the 1 h exposures in Fig. 1 to enable comparison with values for the second generation given above.

4. Discussion

First we consider animals growing in Vietnam as a prelude to discussing laboratory results. Thermal conditions in these growth ponds differ markedly from the salterns in San Francisco Bay. Maximum water temperatures where *Artemia* are found growing in the Bay during the summer and fall growing season rarely exceed 24°C, and are almost always several degrees lower (Robert Rofen, personal communication). In contrast, animals in the Vietnam ponds experienced maximum water temperatures near 37°C for most of the 1-month growth period, and the temperature never fell below 25°C during this time (unpublished results). Browne and Wanigasekera (2000) suggested that 30°C is very close to the upper thermal limit for successful reproduction of *A. franciscana* grown in the laboratory. On that basis, *Artemia* growing and reproducing in the Vietnam ponds have clearly escaped this thermal limit. Recall that the first wave of adults in the ponds came directly from development of SF inoculated cysts, so it seems likely that larval, immature and mature adult stages with high thermal tolerance were being selected during growth in the ponds. It is very unlikely that selection operated directly on the inoculated cysts since their temperature tolerance is above 40°C (Sorgeloos, 1980). It is possible that acclimation might be involved in the survival of immature stages and the reproductive success of adults in the ponds, notably since *A. franciscana* has been reported to exhibit the highest phenotypic plasticity of any species in this genus (Browne and Wanigasekera, 2000). However, results from the laboratory studies indicate that natural selection for high temperature tolerance plays the dominant role. Next we discuss results on adults raised in the laboratory from SF cysts used for the inoculation and from cysts produced in Vietnam. It is important to realize that the cysts produced in Vietnam are much more resistant to high temperatures than are cysts produced in San Francisco Bay (Clegg et al., 2000).

First generation adults raised from V cysts are more heat-tolerant than those from SF cysts (Fig. 1) even though cultured in the laboratory under precisely the same conditions and being of the same age. Laboratory culture temperatures (21±1°C) were close to those existing in SF, but very much lower than temperatures in the Vietnam ponds. In other words, V cysts retained the developmental potential to produce heat-resistant adults in the laboratory, regardless of the lower environmental temperature experienced during larval, immature-stage and adult development, over a period of 2–3 weeks, and involving a dozen or so molts. Even more important is the observation that these differences in heat resistance were retained in the second generation of SF and V adults, both produced under the same laboratory conditions using cysts produced by first generation SF and V adults (Section 3.4.). That observation suggests strongly that the greater heat resistance of V adults has a genetic basis, originating in the females and the

cysts they produced in Vietnam, and incorporated into the developmental program of encysted embryos. Differences in the rates of development of SF and V cysts (Section 3.3) are consistent with this interpretation since V cysts take a longer time than SF cysts to produce larvae in cultures where the temperatures are well below those existing in Vietnam ponds.

Frankenberg et al. (2000) found that second generation V adults did not retain the level of heat resistance shown by the first generation. However, in that study second generation adults were not produced from the harvest and culture of cysts, but from nauplius larvae produced directly by first generation females. Thus, encysted diapause embryos were not involved, suggesting that cyst production might play a role in adult thermal tolerance. That is, the degree of thermal tolerance exhibited by adults could be strongly influenced by whether they develop from cysts or from directly-developing embryos that bypass diapause. That possibility deserves further study.

Enhanced resistance to high temperatures in first generation V adults is not reflected in their content of hsp70, compared to the levels in SF adults (Fig. 2B), indicating that factors other than these proteins must be involved. That result was surprising since this family of stress proteins plays a major role in the heat resistance of a wide variety of organisms (Bukau and Horwich, 1998; Karlin and Brocchieri, 1998; Kiang and Tsokos, 1998; Feder, 1999; Feder and Hofmann, 1999; Krebs, 1999; Nollen et al., 1999). Although heat resistance (Fig. 1) and hsp70 levels were not correlated in untreated SF and V adults (controls, Fig. 2B) we observed a strong up-regulation of these proteins 24 h after heat shock in both groups (Fig. 2B) and at the same time that induced thermotolerance was observed (Section 3.1). The latter outcome is not surprising in view of the abundant literature on the heat shock response and induced thermotolerance (Feder and Hofmann, 1999). However, no appreciable differences in the degree of hsp70 up-regulation are seen between SF and V adults, supporting the idea that factors other than these proteins are involved in the differences between the heat resistance of SF and V adults.

Most research on the hsp70 family has focused on cellular and molecular aspects, but there is growing interest in the organismic, evolutionary and ecological aspects of this protein family and the stress response in general (reviewed by Feder and Hofmann, 1999; also see Coleman et al., 1995; Norris et al., 1995; Chapple et al., 1998; Hightower, 1998; Clegg et al., 1999; Feder, 1999). Recent studies by Krebs (1999) on hsp70 in larvae and adults of three species of *Drosophila* from different thermal environments, and by Tomanek and Somero (1999) on marine snails from different locations in the intertidal zone, illustrate the value of this approach, while also uncovering the difficulty of establishing causal connections between levels of stress proteins, thermotolerance and ecological setting (also see Chapple et al., 1998). Because of its habitat diversity, availability and well-known biology, *Artemia* provides a useful system for such studies.

An important difference between cysts and adults is the presence in cysts of very large amounts of the molecular chaperone p26, and the stress protein artemin, neither protein being detected in adults (Fig. 2). Curiously, all attempts to induce their synthesis in other stages of the life cycle have failed (Clegg et al., 1999; Liang and MacRae, 1999; our unpublished results). If p26 is the potent molecular chaperone we believe it to be, then why do adults fail to synthesize this protein when under stressful conditions? It is

possible that the presence of this protein in high amounts interferes with processes ongoing in adults, but absent in cysts, DNA synthesis and cell division being likely candidates (reviewed by Clegg and Conte, 1980). Such 'deleterious effects' of heat shock proteins have been noted in several other systems (reviewed by Feder and Hofmann, 1999).

Comparison of LT_{50} values reveals marked differences in thermal tolerance of *A. franciscana* life history stages. Miller and McLennan (1988) found that hydrated cysts are very tolerant to heating (LT_{50}~49°C, 1-h exposures), and first stage larvae less so (LT_{50} ~42°C, 1-h exposures). Adults cultured from SF cysts had an LT_{50} slightly above 38°C, using only 30-min exposures (Frankenberg et al., 2000) and would be much lower if 1-h exposures were used (Fig. 1). Differences between adult and cyst thermotolerance might involve the presence in cysts of large concentrations of the compatible solute, trehalose (see Clegg and Conte, 1980) well known for its stabilizing properties (see Yancey et al., 1983; Crowe et al., 1992, 1996). Like artemin and p26, trehalose is not present in adult *Artemia*.

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