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Comparisons of stress proteins and soluble carbohydrate in encysted embryos of *Artemia franciscana* and two species of *Parartemia*

James S. Clegg [a,*], Veronica Campagna [b]

[a] *Bodega Marine Laboratory and Section of Molecular and Cellular Biology, University of California, Davis, Bodega Bay, CA 94923, USA*
[b] *Department of Environmental Biology, Curtin University of Technology, GPO Box U1987, Perth, W. Australia, 6845*

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Abstract

We compared stress proteins (p26, artemin, hsp70) and alcohol-soluble carbohydrates (ASC) in cysts of *Artemia franciscana* and two as yet un-named species populations of *Parartemia*, the brine shrimp endemic to Australia. The small stress proteins and molecular chaperones, p26 and artemin, previously thought to be restricted to *Artemia*, and present in very large amounts in its encysted embryos (cysts), were also detected by western blotting in *Parartemia* cysts, even though roughly 85–100 million years have passed since these genera diverged. We interpret this finding as further evidence for the adaptive importance of these proteins in coping with the severe stresses these encysted embryos endure. As expected, hsp70 was present in all three groups of cysts, but apparently at somewhat lower concentrations in those of *Parartemia*. Based on measurements of ASC we propose that the disaccharide trehalose, critical for desiccation tolerance in many animal cells, has probably also been maintained in the metabolic repertoire of *Parartemia* whose cysts have well developed tolerance to severe desiccation.

Keywords: *Artemia*; *Parartemia*; Stress proteins; p26; Artemin; hsp70; Soluble carbohydrate; Cysts

1. Introduction

Massive amounts of two small stress proteins, artemin (De Herdt et al., 1979; De Graaf et al., 1990) and p26 (Clegg et al., 1994) are present in stress-resistant encysted embryos (cysts) of the primitive crustacean and model system, *Artemia franciscana*. Both proteins have been studied reasonably well since those original descriptions (Clegg et al., 1995, 1999; Liang et al.,1997a,b; Willsie and Clegg, 2002; Chen et al., 2003; Crack et al., 2002; Tanguay et al., 2004; Warner et al., 2004). Each protein makes up 10–15% of the total non-yolk protein of these embryos, and neither has been detected in any other life cycle stage beyond the first day or two of larval life (Jackson and Clegg, 1996; Crack et al., 2002). There is good evidence that p26 plays an important role as a molecular chaperone of proteins in this exceptionally stress-resistant embryo (see above references, and review by Clegg and Trotman, 2002). Furthermore, recent work suggests that artemin could be a molecular chaperone for RNA (Warner et al., 2004) adding to the evidence for RNA chaperones, a subject of increasing interest (reviewed by Lorsch, 2002; Henics, 2003). Because p26 and artemin are apparently such important components of the adaptive repertoire of *Artemia* encysted embryos we previously examined a wide variety of invertebrates for p26 and artemin, including the resting stages of other closely related crustaceans. However, neither protein was detected in any of these samples (unpublished survey results). Thus, p26 and artemin were not detected by western blotting in cysts of the fairy shrimp *Branchinecta sandiegoensis* (supplied by Dr. Marie Simovich and Jake Moorad) a closely related, sister species of *Artemia* (see Spears and Abele, 2000) and cysts of another fairy shrimp *Streptocephalus proboscideus*, (supplied by Dr. Luc Brendonck). Not surprising were the negative results obtained using cysts of the more distantly related notostracan, *Triops* sp., and ephippia of the cladoceran, *Daphnia* sp. A caveat in all these studies is the well-known poor cross-reactivity of antibodies against small heat shock proteins (Arrigo and Müller, 2002; Sun and MacRae, 2005). Nevertheless, until the present study there was no evidence that artemin or p26 existed outside the genus *Artemia*.

* Corresponding author. Tel.: +1 707 875 2010; fax: +1 707 875 2009.
E-mail address: jsclegg@ucdavis.edu (J.S. Clegg).

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The genus *Parartemia*, endemic to Australia, is believed to have evolved directly from *Artemia*, or from a common ancestor, with the estimated divergence of these genera taking place approximately 85 to 100 million years ago during the geological isolation of Australia in the late Mesozoic (Williams and Geddes, 1991; Coleman et al., 1998, 2001; Remegio et al., 2001; Van Stappen, 2002). In view of the abundance and importance of p26 and artemin in *Artemia*, we wondered whether these proteins had been maintained in *Parartemia* cysts, and undertook that study. As background, we summarize next some well-known features of *Artemia* cysts and include what little is known about those features in the cysts of *Parartemia*.

The genus *Artemia* is found in harsh, hypersaline habitats world-wide and its biology is well known (see books by Persoone et al., 1980; Decleir et al., 1987; MacRae et al., 1989; Warner et al., 1989; Browne et al., 1991; Abatzopoulos et al., 2002). We primarily use the species *A. franciscana* collected from salterns in the San Francisco Bay of California (SFB) but also found in the Great Salt Lake, Utah, and other locations (Van Stappen, 2002; Abatzopoulos et al., 2002). Fertilized eggs either develop directly into free-swimming larvae that are released from females, or stop developing at the gastrula stage with the embryos now being surrounded by complex shells, and often referred to as "cysts." Encysted gastrula embryos, composed of about 4000 cells (nuclei) enter diapause, a condition of developmental arrest, characterized by a level of metabolic activity so low in these embryos that its detection is a major problem (Clegg and Jackson, 1998; Clegg and Trotman, 2002).

Desiccation is often a normal part of this developmental pathway and is one of the conditions that can terminate diapause, restoring metabolism and development under permissive conditions of water content, molecular oxygen and temperature (reviewed by Clegg and Trotman, 2002). These embryos are among the most resistant of all multicellular eukaryotes to environmental insults, surviving continuous anoxia for *years*, while fully hydrated at physiological temperatures (Clegg, 1997), temperature extremes, as well as severe desiccation, with cycles of dehydration/re-hydration, and exposure to various forms of radiation (reviewed by Clegg and Trotman, 2002; also see Tanguay et al., 2004). Those features, plus commercial availability in large amounts, make them a useful model system in which to study the biochemistry of resistance to extreme stress in cells that I have referred to as animal extremophiles (Clegg and Trotman, 2002; Abatzopoulos et al., 2002).

The majority of *Parartemia* species are restricted to south western Australia (Williams and Geddes, 1991). The high incidence of inland salt ponds and lakes, and their antiquity and isolation in Western Australia, has resulted in a remarkable radiation of this genus (Geddes, 1981; Remegio et al., 2001) with most species restricted to this area. It is expected that more species will be identified within the family Parartemiidae.

The general characteristics of *Parartemia* cysts, and their developmental pathways, appear similar to those of *Artemia*, but we know little about the details. Although morphologically similar, the cysts of *Parartemia* differ from those of *A. franciscana* from the SFB in that the former sink to the sediment where they remain until hatching takes place. In contrast, most cysts of *A. franciscana* float after being released from females into the hypersaline environment.

2. Materials and methods

2.1. Sources of Artemia and Parartemia cysts

Dried encysted gastrula embryos (cysts) of *A. franciscana* from the South San Francisco Bay salterns were purchased from San Francisco Bay Brand, Hayward, California in 1982 and have been stored dry, under nitrogen gas at about −10 °C, since then. The viability (hatching level) of these embryos has remained close to 90% to the present time. Before use, the dried frozen embryos, still under nitrogen gas, were equilibrated at room temperature for 5 days. Hatching assays were performed as described (Clegg, 1997) in filtered aerobic seawater (SW) at room temperature (~22 °C) and constant laboratory light.

Parartemia cysts were collected in dry surface sediment from two athalassohaline lakes: Lake Yindarlgooda in September 2001, and from Lake Miranda in April 2003. The sediment was oven dried at 40 °C, sieved through 500–180 μm Endecotte® sieves and then stored in plastic vials at room temperature.

Lake Miranda is a large inland salt lake approximately 900 km NE from Perth, Western Australia, with an average background salinity of 20 g/L and a pH of about 6.3. The lake contains abundant populations of *Parartemia*, whose cysts will be referred to here as species A since it has yet to be named.

Cysts of *Parartemia* species B were collected from Lake Yindarlgooda, also a large temporary inland lake 700 km E from Perth, with an average background salinity of 40 g/L and pH 7.5–8.0. These lakes are approximately 500 km apart and are influenced by anthropogenic secondary salinisation as a result of mining activities, with some sites reaching salinities up to 150 g/L. *Parartemia* sp. A recorded maximum hatching in salinities up to 25 g L^{-1} and an average pH of 8. *Parartemia* sp. B hatched best at an average salinity of 32 g L^{-1}, pH of 7. During both trials the dissolved oxygen levels remained at approximately 7 ppm and the temperature was 20–22 °C. Consecutive repeated rewetting of the sediment resulted in continued hatching of the cyst bank; however, no specific values for the percentage of cysts that hatch were obtained.

Soil samples containing *Parartemia* cysts were shipped to the Bodega Marine Laboratory where they were subjected to brief floatation in saturated NaCl. Most of the soil particles sank, and the floating cysts were skimmed off and placed on filter paper to absorb interstitial NaCl solution. These cysts were then rinsed quickly with distilled water and blotted with filter paper. After air drying, individual cysts, along with an unavoidable small amount of debris, were collected with a sharpened wooden splint and pooled until sufficient material was collected for study.

Unfortunately, no hatching assays were carried out on the *Parartemia* cysts used in this study, so the percentage of dead (non-hatching) cysts is not known. The latter could be involved with the lower protein content of *Parartemia* cysts. However, we believe that the smaller amounts of protein in these cysts on a dry weight basis, compared to cysts of *A. franciscana*, are