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Differences of Shell Structure in Cysts of *Artemia* from Various Depth of Urmia Lake (Iran)

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Abstract: *Artemia* is a branchiopod crustacean that inhabits hypersaline habitats and has the ability to produce dormant embryo in gastrula state with tertiary membrane that named cyst. The main source of *Artemia* in Iran is Urmia Lake. The present study under taken to compare ultra structure of shell in 2 different groups *Artemia urmiana* cysts with different ability in buoyancy and role of shell layer in buoyancy or sinking. Cysts of *Artemia urmiana* collected from 2 levels of water (surface and bed) and for electromicroscopy fixed in glutaraldehyde and osmium tetroxide and dehydrated by graded series of ethanol and propylene oxide. Then, they embedded in resin (Agar 100) and 70-80 nm sections colored in 2 stages by uranyl acetate and lead citrate. Ultra structural observation showed some differences in thickness of alveolar (4.5-16 μ) and fibrous (5.5-0.65 μ) layers. Results as indicated from structural differences, showed shell layers are effective in buoyancy or sinking of cysts and against previous ideas about non-relation between buoyancy and shell layers thickness, confirm that cysts set in different depths based on their thickness specially alveolar layer.

Key words: *Artemia urmiana*, cyst, shell structure, electromicroscopy, Urmia Lake

INTRODUCTION

Artemia is branchiopods crustacean that inhabit hypersaline habitats in inland salt lakes, costal lagoons and salterns at tropical, subtropical and temporal regions with the exception of Antarctic (Van Stappen *et al*., 2001; Eimanifar *et al*., 2006). They have been recorded in over 600 costal and inland sites worldwide (Triantaphyllidis *et al*., 1998; Van Stappen *et al*., 2001). The genus of *Artemia* consists of a set of 8 bisexual species defined by the criteria of reproductive isolation as *A. franciscana* (Kellogg, 1906), *A. persimilis* (Piccinelli and Prosdocimi, 1968), *A. monica* (Lenz, 1980), *A. salina* Leach (Triantaphyllidis *et al*., 1997), *A. urmiana* (Gunther, 1890), *A.sinica* (Cai, 1989), *Artemia* sp. from Kazakhstan (Pilla and Beardmore, 1994) and *A. tibetiana* (Abatzopoulos *et al*., 1998) and a large number of parthenogenesis populations can be a great variety of ploidies as di-, tri-, tetra-, penta-and heteroploid grouped under the binomen *A. parthenogenetica* (Sun *et al*., 1999). *Artemia* have the ability to produce dormant embryos, or cysts, that readily available commercially in large quantities and can easily be raised in the laboratory to produce nauplius larvae and adults (Bagshaw, 1989).

From the early blastula stage to the point of hatching, four membranes surround the embryo: the cyst shell (tertiary membrane), the embryonic cuticle (membrane), the hatching membrane and the larval (naupliar) cuticle of the first naupliar instar. The encysted embryo is in the post gastrula state at the time of release from the cyst. The outermost layer is the cyst shell. This rigid structure only found on embryos that are ovoviviparous and it is not secret around embryos that develop in uterus (Macrae *et al*., 1989). Anderson *et al*. (1970) demonstrated that the components of the tertiary membrane are secreted by the shell glands and come to surround the blastula while it is in the uterus. The super structure of the tertiary membrane appears to develop from the inside out after contact with the embryo.

The main source of *Artemia* in Iran is Urmia Lake, located in the northwest of Iran, the *Artemia* live in this lake named *Artemia urmiana* and it is a bisexual species.

Preliminary studies on the structure of the chorion of *Artemia* cysts from Urmia Lake and Great Salt Lake (USA) have indicated differences in relative proportions of the various chorion layers of cysts from these populations which could provide an insight in to the basis of the differences in the buoyancy of these cysts (Sorgeloos, 1997). Whereas, Grate Salt Lake (GSL) samples are floating from 100 ppt onwards soon after incubation, this is only very partially the case for most *Artemia urmiana* samples. Even at the highest salinity a large amount of cysts sink or remained suspended in the water column. Comparative testes showed that as San Francisco

Bay (SFB) samples floated in all experimental salt solution (35-200 ppt), most cysts of the Iranian strain sank to the bottom at salinities below 150 ppt (Sorgeloos, 1997). These laboratory data confirmed by numerous observations in the field, which indicated that *Artemia urmiana* cysts have a tendency of remaining floating in surface or suspended in the water column and sinking to the bottom, more so than cysts from SFB and GSL (Sorgeloos, 1997).

It is not clear if this difference related to the production season, or to environmental parameters, or is inherent to the Urmia strain due to a deviant structure or composition of chorion layers. For this reason, the present study undertaken to compare the ultra-structure of the shell in 1 different groups *Artemia urmiana* cysts with different ability in buoyancy to reveal structural difference between buoyant (cysts accumulated in surface layer) and sink (cysts precipitated in bottom layer) cysts, role of shell in buoyancy or sinking.

MATERIALS AND METHODS

Cysts of *Artemia urmiana* collected from 2 levels of the water (surface and bed) of 3 harvesting sites, including the northern, central and southern regions of the Urmia Lake (Fig. 1). Empty shells or cracked cysts floated away by a brief rinse in distilled water and 30 samples of floating and 30 samples of sinking cysts from each sampling site stored in sodium chloride rich artificial seawater (cysts are not activate by high salt concentration). The cysts of *Artemia* were impermeable to fixatives (Morris and Afzelius, 1967), therefore, they suspended in melted dental wax in aluminum foil cups. The wax was chill rapidly by immersing the cups in ice water. Freehand slices immediately made through the wax and they were plunged into cold fixative.

The cysts first prefixed in 3% glutaraldehyde in Millonig's phosphate buffer (Glavert, 1975) for 4 h, then, were fixed for 2 h in 1% osmium tetroxide in Millonig's phosphate buffer. The fixed and washed specimens take rapidly through a graded series of alcohols (25, 50, 70, 96 and 100%) and into propylene oxide prior to embedding in resin (Agar 100). In resin, the remaining fragments of dental wax floated to the surface and easily were remove, the cysts sank to the bottom of the vials. Sections cut with ultra microtome to 70-80 nm thickness and were taking up on carbon-coated, formvar-covered grids. Most sections were double-stained with saturated aqueous uranyl acetate for 35 min at 60°C followed by lead citrate for 10 min at room temperature. Microscopy was performing in a Philips 208 electro microscope.

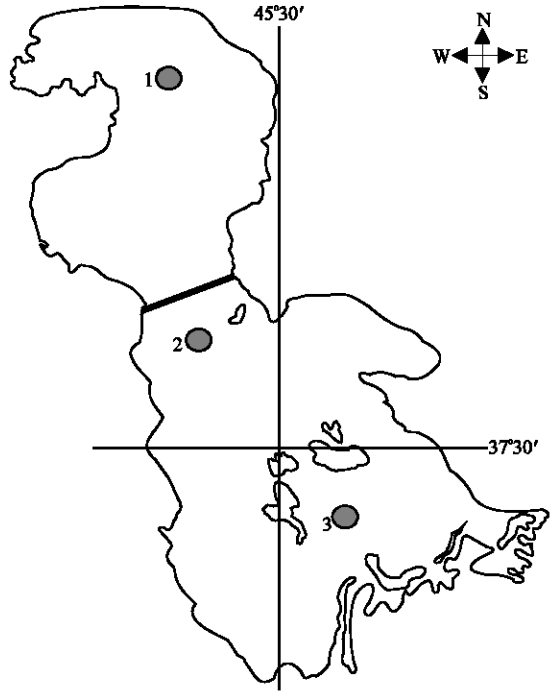

Fig. 1: Sampling site in different geographic parts of Urmia Lake with different rivers, indicated as 1: North area, 2: Median area, 3: South area

RESULTS

Ultra structural observations have shown that 2 main regions of the shell were observed in sections (Fig. 2 and 3). The outer region contains the dark pigment, appears alveolar and is of maternal origin. The inner region is lighter and electron transparent contains less obvious structure and apparently is of embryonic origin. These regions referred to as the chorion and the embryonic cuticle (Morris and Afzelius, 1967). Between chorion and embryonic cuticle is a thin layer, named cuticular membrane. Sufficient information is not available for this layer to include it with the maternally produced chorion or embryonically produced cuticle.

The cortical layer of chorion: This layer (about 0.7 μ thick, Fig. 4) consisted of a dense matrix with radially oriented pores (up to 300 nm wide) in center of layer where they aligned within a system of electron dense parallel lines. The lines extended the width of the cortex and separated from each other by a 250-300 nm gap (Fig. 4). Some specimens contained a thin waxy covering on the outer surface of the cortex, treatment of cysts with alcohol or acetone did not remove this covering.

The alveolar layer of the chorion: This broad layer was represented by a complex system of interconnecting chambers which appeared empty in fixed specimens. The

alveoli intercommunicated either by being confluent or by way of a network of small straight tubules of diameters comparable to those of the radial pores in the cortex (Fig. 4). In favorable specimens, continuity was evident through the pores and alveoli from one surface of the layer to the other. At the innermost surface of the alveolar layer was a 0.12 μ layer of matrix that never contained alveoli or pores (Fig. 4). There was a difference in thickness of alveolar, 4.5-16 μ in surface and bed samples (Fig. 2 and 3).

The outer cuticular membrane: This thin membrane (0.12 μ) consisted of what appeared to be a typical triple-layered biological membrane on the outside separated by a relative wide amorphous layer from a complex inner membrane. This layer resembled two asymmetrical triple-layered membranes in apposition as mirror image (Fig. 5). The thickness, location and resistance to chemical attack, make it similar to the selectively permeable "amber layer" described by Beament in eggs of the bug *Rhodinus prolixus* (Beament, 1946).

The fibrous layer of the embryonic cuticle: In many ways, this layer resembled the fibrous endocuticle of the crustacean exoskeleton. Similar to, the endocuticle, the fibrous layer contains chitin and protein, deposited by the underlying cells, divided into polygonal primes or platelets by radial septa and composed of fibrous lamellae

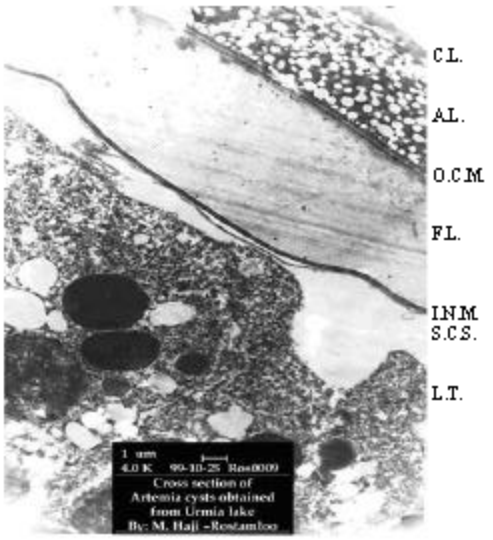

Fig. 2: Cross section of *Artemia urmiana* cysts (sank samples): C.L. = Cortical Layer, A.L. = Alveolar Layer, O.C.M. = Outer Cuticular Membrane, F.L. = Fibrous Layer, I.N.M. = Inner Cuticular Membrane, S.C.S. = Subcuticular Space, L.T. = Larval Tissue

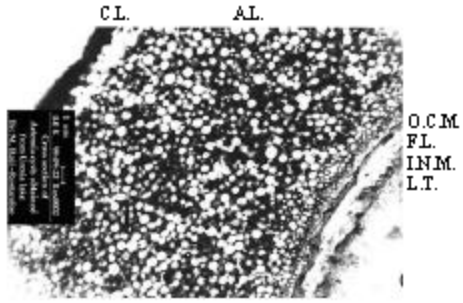

Fig. 3: Cross section of *Artemia urmiana* cysts (buoyant samples): C.L. = Cortical Layer, A.L. = Alveolar Layer, O.C.M. = Outer Cuticular Membrane, F.L. = Fibrous Layer, I.N.M. = Inner Cuticular Membrane, S.C.S. = Subcuticular Space, L.T. = Larval Tissue

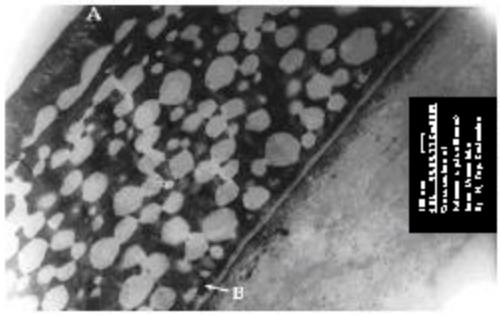

Fig. 4: The cortical and alveolar layers of *Artemia urmiana* cysts: A= dense matrix with radially oriented pores, B= layer of matrix that never contained alveoli or pores

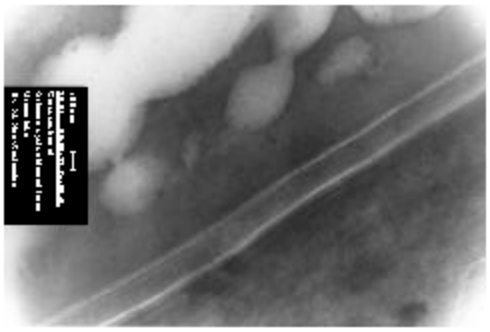

Fig. 5: The outer cuticular membrane of *Artemia urmiana* cysts

(Fig. 6). There was a difference in thickness of fibrous, 0.65-5.5 μ in surface and bed samples (Fig. 2 and 3).

The inner cuticular membrane: This is the innermost layer of the shell and separated from the underlying cells by subcuticular space (Fig. 6). No fine structure observed

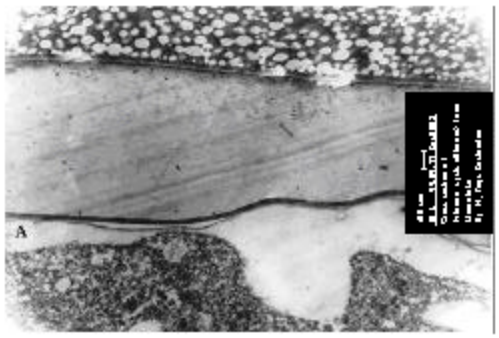

Fig. 6: The fibrous layer of *Artemia urmiana* cysts: A= subcuticular space

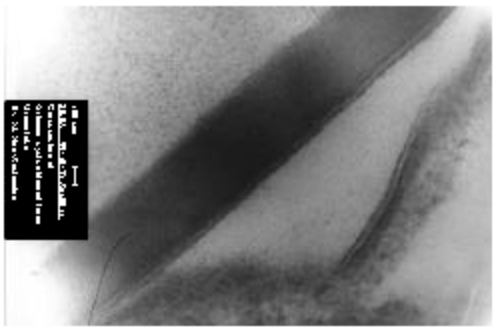

Fig. 7: The inner cuticular membrane of *Artemia urmiana* cysts

within this membrane and there was a little structural definition. It quite consistently had the same thickness over the entire embryo, about 0.21 μ (Fig. 7). A thin non-staining layer and a thin dense layer bound it internally, respectively. In some specimens, the subcuticular space contained many small particles which judging by their size and shape either small glycogen particles or ribosome.

Embryos were in gastrula state in all cysts and it was same in both buoyant and sank cysts with no difference.

DISCUSSION

The function of the tertiary membrane thought to be more physical than chemical since many components can pass through this layer (inner membrane acts as a semi-permeable barrier). It is also suggested that certain compounds in this layer might act as chemical signals to direct the embryonic cells towards the developmental pathway of encystment and anhydrobiosis. Hydration and development changed the shell structure very little. Normal development followed the removal of the large and complex chorion by hypochlorite and this layer offered no resistance to the exchange of ions. Thus, it may be assumed that if the chorion had some function in the life of the cyst. It should be before the cysts were rehydrated (Macrae *et al*., 1989).

Figure 8 summarizes the foregoing description diagrammatically. The cross sections revealed some differences in the relative proportions of the various chorion layers for 2 different groups: thinner alveolar layer and thicker fibrous layer for buoyant samples. The consistently spherical shape of the alveoli and their lack of electron-dense content suggest that they formed by gas bubbles. Assuming this is the case, the alveolar layer may act as a float for cyst. Such flotation mechanisms consisting of low density liquid or gas at or below the atmospheric pressure are known from a variety of organisms (Morris and Afzelius, 1967). Because cysts and shells are extremely hygroscopic (Morris and Afzelius, 1967), water must be excluded from the cysts if the shell is to act as a flotation device. One means of doing this suggested by the remnants of membranes covering some cysts. Assuming that the developing cysts were dehydrated in the brood sac by an osmotic pump or some other mechanism such as the replacement of water by gas, the outer membrane would serve to maintain the inactive state known in the diapause cysts before they have been dried in air. The fibrous layer contains chitin and protein, has considerable density and may act as a sink for cyst. It seems to be existence of thin fibrous layer is the second reason for flotation in buoyant cysts. Previous observations (Sorgeloos, 1997) were only made for a single batch of *Artemia urmiana* cysts. Results showed that there was non-relation between buoyancy and shell layers thickness and it remains to be further investigation for the behavioral consequences of these structural differences. Our results as indicated from structural differences, showed shell layers are effective in buoyancy or sinking of cysts and against previous ideas about non-relation between buoyancy and shell layers thickness. Thus, confirms that cysts set in different depths based on their thickness especially alveolar layer.

One hypothesis for explaining these differences is that there are 21 permanent and seasonal rivers (annual inflow 94.9×10^9 m^3) flowing through agriculture, urban and / or industrial areas (Fig. 1), draining in to this terminal lake predominantly in to its southern area, largely without waste water treatment (Ghaheri *et al*., 1999). These rivers have different chemical and physical properties and an unequal density of water has appeared in the different geographical areas. Seasonal variations will also affect this phenomenon. Our investigations indicate that in spring the concentration of lake water is heterogeneous (because of melting ice and increasing of super facial flow of waters), but in fall the concentration of lake water is

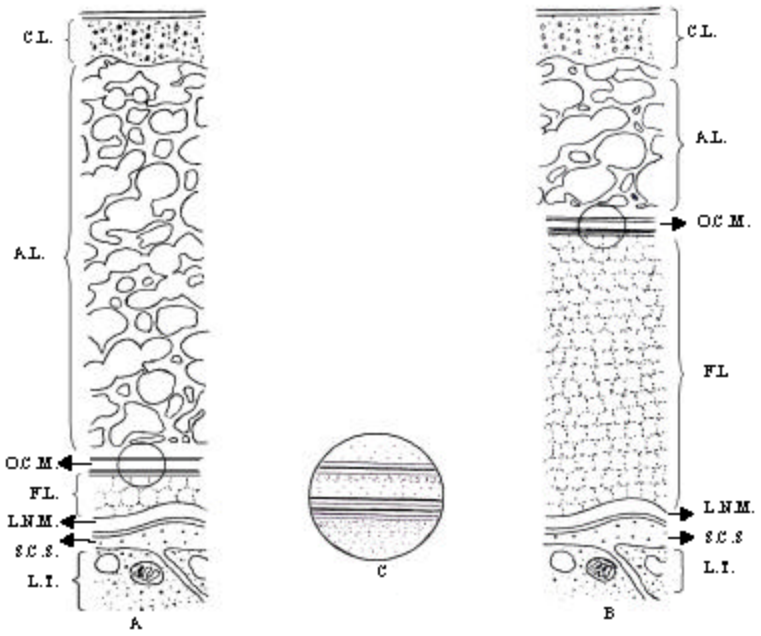

Fig. 8: Composite diagrams of shell and membranes in buoyant (A) and sink (B) *Artemia urmiana* cysts and detail of the outer cuticular membrane (C): C.L. = Cortical Layer, A.L. = Alveolar Layer, O.C.M. = Outer Cuticular Membrane, F.L. = Fibrous Layer, I.N.M. = Inner Cuticular Membrane, S.C.S. = Subcuticular Space, L.T. = Larval Tissue

homogenous and causes a great mass transfer between mentioned zones which in return can affect environmental aspects. The differences in salinity between northern and southern areas of the lake limited (5-10 g L^{-1} lower in southern area as a consequence of river flow) and tend to become minimal in winter months (Van Stappen *et al*., 2001). These ecological changes, affect the structure of the lake and probably different populations have formed in various ecological areas of Urmia Lake.

On the other hand, comparison between cross section and thickness of different layer in cysts of *A. urmiana* and *A. salina* (Macrae *et al*., 1989; Morris and Afzelius, 1967) indicated that *A. urmiana* averagely have thinner alveolar layer and thicker fibrous layer than *A. salina*. Whereas, *A. salina* samples are floating from 100 ppt, even at the highest salinity a large amount of *A. urmiana* cysts sinks or remains suspended in the water column and most cysts of the Iranian strain sank to the bottom at salinities below 150 ppt (Sorgeloos, 1997). It is clear that in spite of differences in size, both *A. urmiana* and *A. salina* cysts have similar structure that indicated to derivation of *A. urmiana* from *A. salina* (Sorgeloos, 1997).

CONCLUSION

The cross sections revealed some differences in the relative proportions of the various chorion layers for 2 different groups: thinner alveolar layer and thicker fibrous layer for buoyant samples. Assuming this is the case, the alveolar layer may act as a float for cyst. The fibrous layer contains chitin and protein, has considerable density and may act as a sink for cyst. It seems to be existence of thin fibrous layer is the second reason for flotation in buoyant cysts.

Previous observations (Sorgeloos, 1997) were only made for a single batch of *Artemia urmiana* cysts. Results showed that there was non-relation between buoyancy and shell layers thickness and it remains to be further investigation for the behavioral consequences of these structural differences. Our results as indicated from structural differences, showed shell layers are effective in buoyancy or sinking of cysts and against previous ideas about non-relation between buoyancy and shell layers thickness. Thus, confirms that cysts set in different depths based on their thickness especially alveolar layer.

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