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Original Article

Morphology and Ultrastructure of cysts in different species of the brine shrimp, *Artemia* from Southern India

Shanmugam Sivagnanam[1], Velayudhannair Krishnakumar*[2], Natesan Munuswamy[2]

[1]Central Institute of Brackishwater Aquaculture, Santhome High Road, Chennai – 600 028, India.
[2]Unit of Aquaculture and Cryobiology, Department of Zoology, University of Madras, Life Science Building, Guindy Campus, Chennai – 600 025, India.

Abstract: Surface topography and ultrastructure of dried cysts of *Artemia parthenogenetica* (Vedaranyam population) and bisexual *Artemia* sp. (Kelambakkam population) were studied electron microscopically in order to identify the bisexual strain, inhabiting Covelong salterns, Kelambakkam, South India. The scope of this study is to provide substantial information for further characterization of these *Artemia* strains by the comparison of the cyst morphology and ultrastructure. A cyst of *Artemia franciscana* was used as a reference cyst for comparison. Scanning electron microscopic studies on cyst morphology revealed that the surface is smooth with no significant variation among the three *Artemia* strains studied. Transmission electron microscopic observations on the ultrastructure of the cyst both parthenogenetic and bisexual forms showed an apparent variation in thickness of the cortical layer. In Kelambakkam population of *Artemia*, the architecture of alveolar meshes is tightly arranged and is similar in shape. But they show minor variations in the arrangement of pores in the matrix and in the length of the cortical layer, compared to *A. franciscana*. In *A. parthenogenetica*, they are loosely arranged and are oval in shape. The present study clearly documents that the *Artemia* species, colonized in Kelambakkam saltpan is *A. franciscana*; thus, the variation in the cyst ultrastructure is much pronounced and a taxonomically important parameter for the genus *Artemia*.

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Introduction

Representatives of the branchiopod crustacean, the brine shrimp *Artemia* spp. inhabit hypersaline habitats (Van Stappan et al., 2001). The genus *Artemia* consists of several bisexual and parthenogenetic forms which probably diverged five to six million years ago from a common ancestor living in the Mediterranean area (Abatzopoulos et al., 2002; Munoz et al., 2010) and the latter with a variety of ploidies (Sun et al., 1999). It is well known that the reproductive pattern of *Artemia* switches from ovoviviparity (Browne, 1980) to oviparity (Clegg et al., 1996) depending on the environmental factors in the habitat. Brine shrimps can produce dormant eggs, covered by a characteristic shell or chorion under adverse environmental conditions such as high salinity, low dissolved oxygen and nitrate content (Krishnakumar et al., in press). The shell around the egg is secreted by a cluster of shell glands. The shell itself plays an important role in the protection of the embryo (Anderson et al., 1970) in both, parthenogenetic and bisexual species.

Encystment (cyst formation) protects the embryo from various extreme environmental conditions and during dormant stage (Clegg, 1974; Versichele and Sorgeloos, 1980). Embryo resumes its development, when the dehydrated cysts are hydrated at suitable environmental conditions, such as suitable osmotic pressure and sufficient oxygen (Dutrieu, 1960). The cyst of *Artemia* consists of several layers (Lee et al.,

* Corresponding author: Velayudhannair Krishnakumar
E-mail address: kv.krishnakumar@hotmail.com
Tel: +919894304073

1994; Rosowski, 1997). Outside the embryonic cuticle, and forming the outer surface of the cyst, there is a thick nonchitinous layer referred to as a 'chorion' (Morris and Afzelius, 1967).

In India, the distribution of *Artemia* has been well documented in the saltpans of various states like Gujarat, Rajasthan, Tamilnadu and Maharashtra (Kulasekarapandian et al., 1992). It was reported that *A. parthenogenetica* was dominated in all the saltpans of South India until the late 1990s. The occurrence and even dominance of invasive bisexual species was reported in a few saltpans in Tamilnadu, including Kelambakkam (Kulasekarapandian et al., 1992; Sivagnanam et al., 2011; Vikas et al., 2012) and Tuticurin (Sugumar and Munuswamy, 2006) due to the improper disposal of *Artemia* cysts used at various shrimp hatcheries to feed the shrimp larvae.

Earlier interest in the study of anostracan cysts led to the description of its structure and hypothecated the importance of cyst ultrastructure during diapausing period and development during the hydration process of the embryo. Later, it became an integrated part of species or strain description (Belk and Sissom, 1992) and species identification keys were developed (Thiery and Gasc, 1991; Brendonck and Coomans, 1994). The structure of cyst and cyst membrane became an important character in species identification for several species (Wurdak et al., 1978; Gilbert et al., 1979; Munuswamy et al., 1996). The present study was undertaken to identify, further characterize and to understand extend of invasion and naturalization of invader *A. franciscana* using the cyst morphology and ultrastructure of different strains of *Artemia* inhabiting various salterns in South India.

Materials and Methods

Sources of Artemia: Cysts of parthenogenetic and bisexual species of *Artemia* were collected from the saltpans of Vedaranyam (VRM) (08º59' N; 78°50' E) and Kelambakkam (KBM) (12°08'N, 80°02'E), South India, respectively, during 2008 to 2011 during available period. Collected cysts were transported to the laboratory in 2 or 3 L of screw-

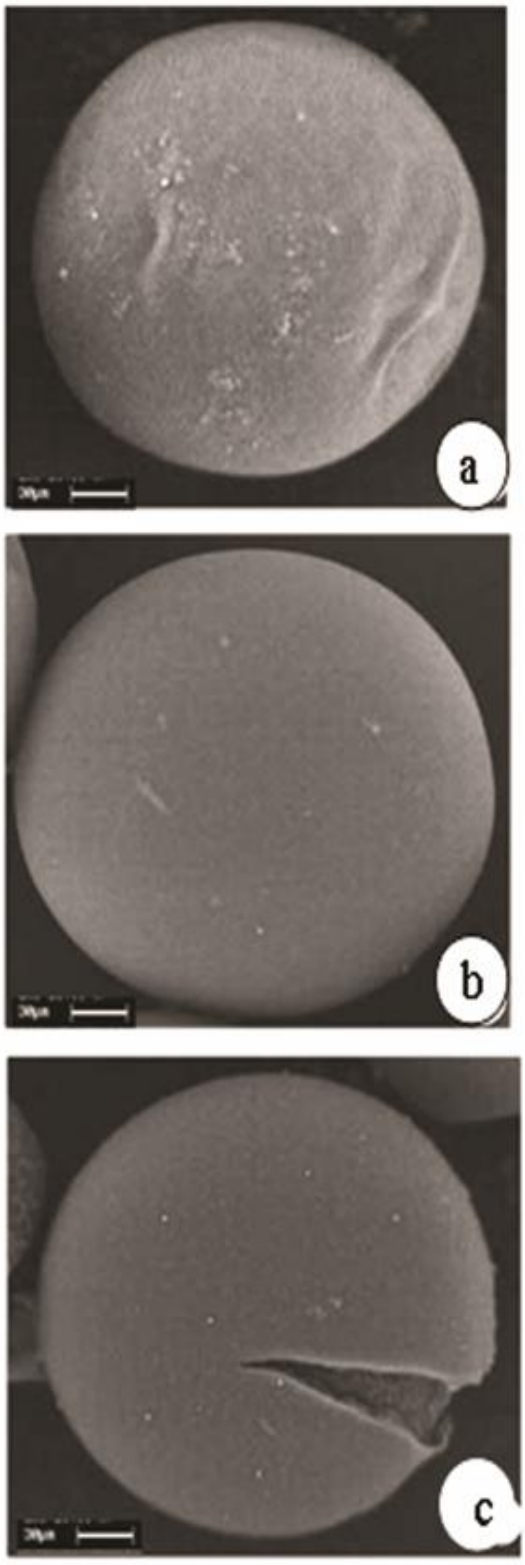

Figure 1. Scanning electron micrographs of the hydrated cyst of different strains of *Artemia*, showing smooth and granular surface. Bar = 30 µm; (a) *A. franciscana* (SFB population), (b) *Artemia* sp. (KBM population) and (c) *A. parthenogenetica* (VRM population).

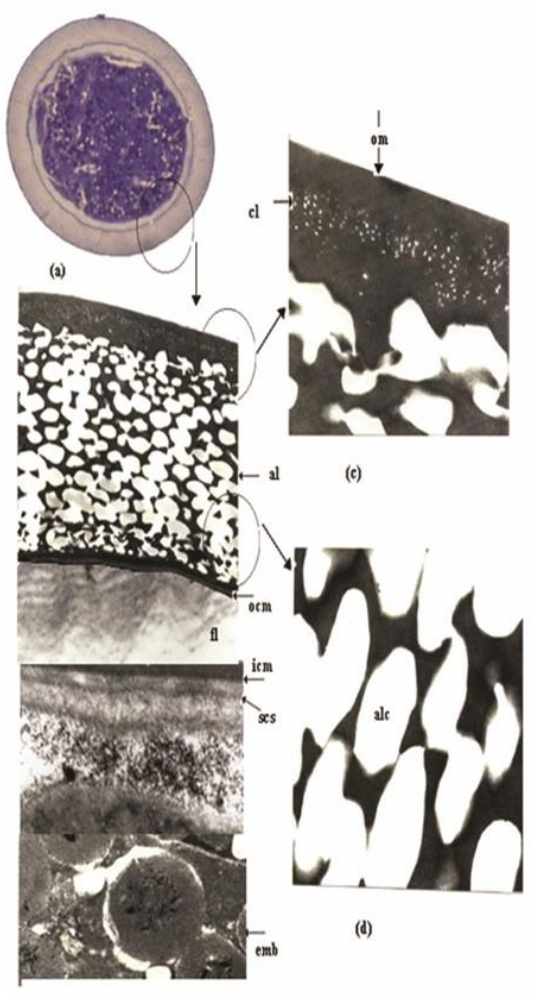

Figure 2. Transmission electron micrographs of *A. parthenogenetica* cyst (VRM population). (a) Ultra-thick section showing the architecture of cyst membranes of an encysted gastrula, (b-d) transmission electron micrographs of tertiary cyst membranes, (c) ultrastructure of outer cortical layer and inner alveolar layer at higher magnification; note the thickness of the cortical layer and (d) alveolar membrane showing alveolar mesh with spongy nature. om- outer membrane; cl- cortical layer; al- alveolar layer; ocm- outer cuticular membrane; fl- fibrous layer; icm- inner cuticular membrane; scs- sub cuticular space. emb-embryo.

capped plastic containers having brine solution (>250 ppt) and washed with freshwater for 2 minutes. Washed cysts were dried in an oven at 37ºC for 24 h and sieved through a 300 µm mesh and packed at vacuum (Sevana's Quick Seal, Hyderabad, India) (Sorgeloos and Kulasekarapandian, 1984). Vacuum packed cysts were stored at room temperature after proper labeling for further analysis. Commercially available cysts of *A. franciscana*, (OSI brand *Artemia* cysts, purchased from M/s. Southern India Pvt. Ltd., Chennai, South India) were used as a reference.

Scanning Electron Microscopy: For scanning electron microscopy, cysts of all the three *Artemia* samples, collected from the salterns of Kelambakkam, Vedaranyam and *A. franciscana* were rinsed with distilled water and immersed in 10% formalin for 30 min to remove the microorganisms and debris attached to the surface of the shell. The cysts were then fixed in 2.5% glutaraldehyde, prepared in cacodylate (sodium phosphate) buffer adjusted to pH 7.4 for several hours. The cysts were washed and post-fixed in 2% osmium tetroxide and then dehydrated in a graded alcohol series. Thereafter, the cysts were air dried for a few minutes at room temperature, critical point dried and glued onto standard mica squares (1 cm^2), mounted on metal stubs, subsequently coated with gold (SEM Leo 435 UP) and viewed and photographed under a scanning electron microscope (Leo Stereo Scan, 440).

Transmission Electron Microscopy: For transmission electron microscopy, dried cysts were fixed in 2% glutaraldehyde prepared in cacodylate (sodium phosphate) buffer (pH 7.4). The cysts were then post-fixed in 1% osmium tetraoxide and processed for electron microscopy. The tissue was embedded in epoxy resin and sectioned using ultra microtome. Ultra-thin sections of the cysts were taken and stained with uranyl acetate and viewed using transmission electron microscope (Philips, 20IC, Netherlands).

Results

Scanning electron micrographs on the hydrated cyst of parthenogenetic and bisexual and exotic (bisexual) species of *Artemia* showed smooth granular surface with no significant difference on the surface topography (Fig. 1). Transmission electron microscopic observations of the cysts showed a general pattern of outer cortex, inner alveolar layer and embryonic cuticle. The outer region is dark pigmented cortical layer followed by large alveolar layer which constitutes the tertiary cyst membrane.

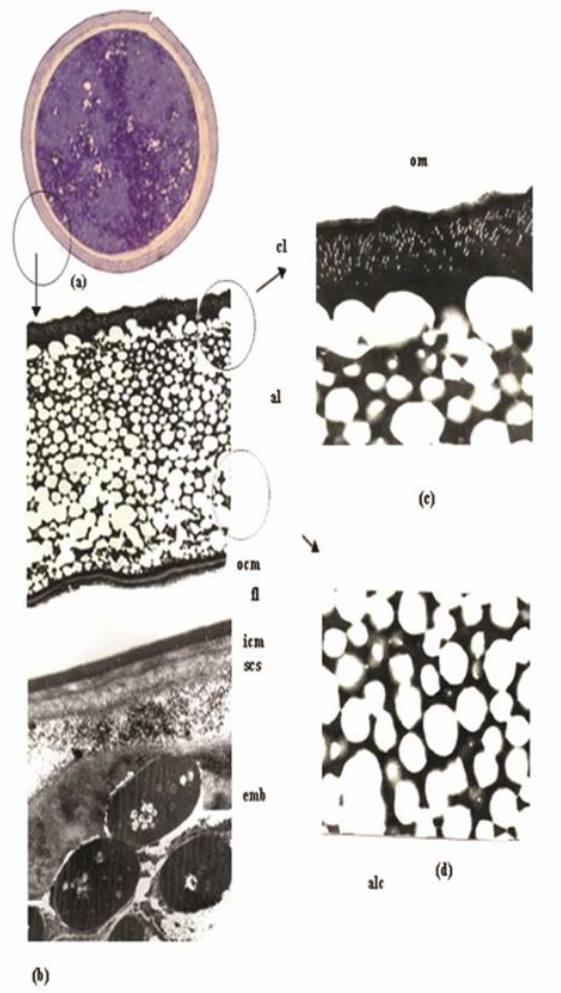

Figure 3. Transmission electron micrographs of *Artemia* sp. cyst (KBM population). (a) Ultra thick section showing the architecture of cyst membranes of an encysted gastrula, (b-d) transmission electron micrographs of tertiary cyst membranes, (c) ultrastructure of outer cortical layer and inner alveolar layer at higher magnification; note the thickness of the cortical layer and (d) alveolar membrane showing alveolar mesh with spongy nature.om- outer membrane; cl- cortical layer; al- alveolar layer; ocm- outer cuticular membrane; fl- fibrous layer; icm- inner cuticular membrane; scs- sub cuticular space; emb- embryo.

The thickness of the outer membrane showed considerable variations between parthenogenetic and bisexual species.

Ultrastructure of outer cortical layer showed dense matrix with small pores arranged as a line in the centre and extended the width of the cortex. These pores are restricted in central matrix of the other cortical layer in *A. parthenogenetica* and it is distributed throughout the width of the matrix in *Artemia* sp. (KBM population). A significant variation in the thickness of cyst membranes is found between bisexual and parthenogenetic species. The cortical region is not tightly bound to the alveolar region in *A. parthenogenetica* and is closely arranged in *Artemia* sp. of both KBM and SFB populations. The outer protective cortex layer is followed by a broad spongy alveolar layer which is characterized by the presence of interconnecting chambers in all the strains. Alveolar layer is much larger in size and tightly packed in *A. parthenogenetica* compared to KBM and SFB populations. In parthenogenetic cysts, the alveolar chambers are oval in shape and are loosely arranged; but in bisexual species, these are tightly attached each other and the chambers are round in shape and conjugated form and seems similar to that of SFB populations (Figs. 2, 3 and 4). The alveolar layer is followed by a fibrous layer, located between the inner and outer cuticular layer. The outer and inner cuticular membranes are thin and appeared as triple layered biological membrane and not much variation noticed among the strains. Embryonic mass is located safe beneath the inner cuticular membrane (Figs. 2, 3 and 4).

Discussion

The cysts of the two different populations (VRM parthenogenetic and KBM bisexual populations) of *Artemia* have been thoroughly examined for morphological and ultrastructural variations. The surface topography of *Artemia* cyst, distributed worldwide, has an externally smooth surface (Anderson et al., 1970; Trotman et al., 1987) except in *A. monica*. In the present study it was observed that the cysts of all the species studied exhibit smooth surface and no considerable variation was observed concerning the cyst surface topography. On the other hand, Sugumar and Munuswamy (2006) observed a button shaped structure on the cyst of Puthalam *Artemia* population apart from its normal smooth surface.

TEM studies showed different layers such cortical layer, alveolar layer, outer cuticular membrane, fibrous layer and inner cuticular membrane. The cross sections showed some difference in the arrangement of pores in cortical layer between

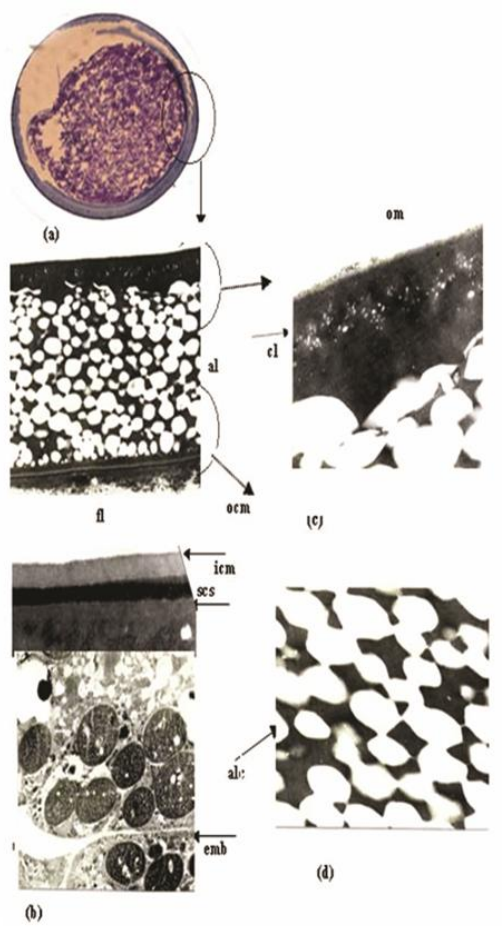

Figure 4. Transmission electron micrographs of *A. franciscana* cyst (SFB population). (a) Ultra thick section showing the architecture of cyst membranes of an encysted gastrula, (b-d) Transmission electron micrographs of tertiary cyst membranes, (c) Ultrastructure of outer cortical layer and inner alveolar layer at higher magnification Note the thickness of the cortical layer and (d) alveolar membrane showing alveolar mesh with spongy nature. om- outer membrane; cl- cortical layer; al- alveolar layer; ocm- outer cuticular membrane; fl- fibrous layer; icm- inner cuticular membrane; scs- sub cuticular space; emb- embryos.

parthenogenetic and bisexual species. The consistently spherical shape of the alveoli suggests that they filled with or formed by gas bubbles, which may assist the cyst to float (Morris and Afzelius, 1967). The outer cover of the *Artemia* cyst consists of an outer surface lamella, alveolar lamellae and a tertiary envelope (Lee et al., 1994) followed by the embryonic cuticle and outer cuticle of the embryo. It is suggested that the function of the tertiary layer might be rather physical than chemical since many components can pass through this layer (Hajirostamloo, 2008). Because of hygroscopic nature, water must be omitted from the cysts if the shell is to act as a flotation device (Hajirostamloo, 2008). The inner network space connection is restricted to the surface of the cyst shell and helps to reduce the water loss from the cysts when exposed to a dry environment. It may also help respiration to gastrula, like plastron respiration to keep it for a long period in live encysted conditions (Clegg, 1974). Similar results on the variation of the alveolar layer in different strains was reported by Abatzopolous et al. (2006) and Sugumar and Munuswamy (2006). Munuswamy et al. (1996) suggested that the difference in alveolar layer and alveolar mesh in rotifers provides a species specific pattern.

Emergence of nauplius from the hydrated cyst is induced by the light and the interpretation of light is based on the thickness of the cyst membrane. Kulasekarapandian reported that parthenogenetic strain had low hatching percentage compared to bisexual strains, this may be due to the variations in the thickness (Abatzopoulos et al., 2006). In the present study, parthenogenetic and bisexual species of *Artemia* showed significant differences in their chorion thickness and such variations may because of the prevailing environmental conditions (such as salinity and ions) at Kelambakkam saltpan. The studies on the cyst topography and ultrastructure of the three *Artemia* populations in the present study demonstrate to a certain extent, that the variations in the alveolar and fibrous layers are more 'suitable' for characterizing *Artemia* populations than whole homogenates of decapsulated cysts.

Our previous study on adult morphology, cyst and naupliiar biometry (Krishnakumar, unpublished data) and protein profiles (Sivagnanam, 2005) suggested that the *Artemia* inhabiting Kelambakkam saltern is like that of *A. franciscana*. The findings of the present study helps to further characterize this strain. Further, the present study suggested that the cyst membrane thickness and difference in alveolar mesh can be considered to be taxonomic tools to characterize *Artemia* strains as in rotifers (Munuswamy et al., 1996) and fairy shrimps (Walsche et al., 1991). This conclusion can also be

better supported by close study of the results obtained with different *Artemia* populations.

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References

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

Abatzopoulos T.J., Baxevanis A.D., Triantaphyllidis G.V., Criel G., Pador E.L., Van Stappen, G., Razavi Rouhani S.M., Sorgeloos, P. (2006). Quality evaluation of *Artemia urmiana* Gunther (Urmia Lake, Iran) with special emphasis on its particular cyst characteristics (International Study on *Artemia* LXIX), Aquaculture, 254: 442-454.

Anderson E., Lochhead J.H., Lochhead M.S., Huebner, E. (1970). The origin and structure of the tertiary envelope in thick-shelled eggs of the brine shrimp *Artemia*. Journal of Ultrastructure Research, 32(5-6): 497-525.

Belk D. Sissom S.L. (1992). New *Branchinella* (Anostraca) from Taxas, USA and the problem of antenna like processes. Journal of Crustacean Biology, 12(2): 312-316.

Brendonck L., Coomans A. (1994). Egg morphology in African Streptocephalidae (Crustacea: Branchiopoda: Anostraca) Part 1: South of Zambezi and Kunene rivers. Archiv Fur Hydrobiologie, 99(3): 313-334.

Browne R.A. (1980). Reproductive pattern and mode in the brine shrimp. Ecology, 61(3): 466-470.

Clegg J.S. (1974). The control of emergence and metabolism by external osmotic pressure and the role of free glycerol in developing cysts of *Artemia salina*. Journal of Experimental Biology, 41: 879-892.

Clegg J.S., Drinkwater L.E., Sorgeloos P. (1996). The metabolic status of diapause embryos of *Artemia franciscana* (SFB). Physiological Zoology, 69(1): 49–66.

Dutrieu J. (1960). Observations biochimiques et physiologiques sur le development d'*Artemia salina* (Leach). Archives of Zoological Experimental Genetics Notes Review, 99: 1–134.

Gilbert J.J., Birky Jr. C.W., Wurdak E.S. (1979). Taxonomic relationships of *Asplanchna brightwelli*, *A. intermedia* and *A. sieboldi*. Archiv fuer Hydrobiologie, 87(2): 224-242.

Hajirostamloo M. (2008). Differences of shell structure in cysts of *Artemia* from various depth of Urmia Lake (Iran). Research Journal of Biological Sciences, 3: 648-653.

Krishnakumar V., Asha A., Sivagnanam S., Munuswamy N (2014). Influence of physico-chemical variates on the distribution of the invader *Artemia franciscana* Kellogg, 1906 (Crustacea: Anostraca) in the salterns of Kelambakkam, Southeast Coast of India, International Journal of *Artemia* Biology (In press).

Kulasekarapandian S., Srinivasagam S., Joseph K.O., Alagarswami K., Rao A.V.P. (1992). *Artemia* cyst production at Kelambakkam, India. In: P. Sorgeloos (Ed), Larviculture and *Artemia* News Letter, 24: 37-38

Lee K.W., Gothro M.A., Belk D., Rosowski J.R. (1994). Ultrastructure features of the tertiary envelope in the cyst of the brine shrimp *Artemia franciscana* (Anostraca). In: G.W. Bailey, A.J. Garratti-Reed, (Ed). Proceedings of the 52nd Annual Meeting of the Microscopy Society of America, San Francisco Press.

Morris J.E., Afzelius B.A. (1967). The structure of the shell and under outer membranes in encysted *Artemia salina* embryos during cryptobiosis and

development. Journal of. Ultrastructure Research, 20: 244-259.

Munoz J., Gomez A., Green A.J., Figuerola J., Amat F., Rico C. (2008). Phylogeography and local endemism of the native Mediterranean brine shrimp *Artemia salina* (Branchiopoda: Anostraca). Molecular Ecology, 17(3): 3160-3177.

Munuswamy N., Hagiwara A., Murugan G., Hirayama K., Dumont H.J. (1996). Structural difference between the resting eggs of *Brachionus plicatilis* and *Brachionus rotundiformis* (Rotifera, Brachionidae): an electron microscopic study. Hydrobiologia, 318(3): 219-223.

Rosowski J.R., Belk D., Gouthro M.A., Lee K.W. (1997). Ultrastructure of the cyst shell and underlying membrane of the brine shrimp *Artemia franciscana* Kellogg (Anostraca) during postencystic development, emergence and hatching. Journal of Shellfish Research, 16(1): 233-249.

Sivagnanam S. (2005). Studies on the characterization and biochemical composition of different strains of *Artemia* (Crustacea: Anostraca) in South India. Ph.D. thesis, University of Madras, India

Sivagnanam S., Krishnakumar V., Kulasekarapandian S., Munuswamy N. (2011). Present status of the native parthenogenetic strain of *Artemia* sp. in the salterns of Tamil Nadu. Indian Journal of Fisheries, 58(4): 61-65.

Sorgeloos P., Kulasekarapandian S. (1984). Production and use of *Artemia* in aquaculture. In: K. Rengarajan (Ed.), Culture of live feed organisms with special reference to *Artemia* culture. Workshop report, CMFRI special publication 15. pp 73.

Sugumar V., Munuswamy N. (2006). Ultrastructure of cyst cell and underlying membranes of three strains of the brine shrimp *Artemia* (Branchiopoda: Anostraca) from the south India. Microscopic Research and Technique, 69(12): 957-959.

Sun Y., Zhong Y., Song W.Q., Zhang R.S., Chen Y. (1999). Detection of genetic relationships among four *Artemia* sp. using randomly amplified polymorphic DNA (RAPD). International Journal of Salt Lake Research, 8: 139-147.

Thiery A., Gasc C. (1991). Resting eggs of Anostraca, Notostraca and Spinicaudata (Crustacea: Branchiopoda) occurring in France: Identification and taxonomic value. Hydrobiologia, 212(1): 245-259.

Tortman C.A.N., Gieseg S.P. Pirie R.S., Tate W.P. (1987). Abnormal development in *Artemia*. Defective emergence of the prenauplius with bicarbonate deficiency. Journal of Experimental Zoology, 243(2): 225-232.

Van Stappan G., Fayazi G., Sorgeloos P. (2001). International study on *Artemia* LXIII. Field study of *Artemia urmiana* (Gunther, 1890) population in Lake Urmiah, Iran. Saline Lakes Developments in Hydrobiology, 162: 133-143

Versichele D., Sorgeloos P. (1980). Controlled production of *Artemia* cysts in batch cultures. In: G. Personne, P. Sorgeloos, O. Roel, E. Jaspers, (Ed), The brine shrimp *Artemia*, Ecology, culturing and use in aquaculture, Vol. 3, Wettern, Belgium, Universa press, pp. 231-246

Vikas P.A., Sajeshkumar N.K., Thomas P.C., Kajal Chakraborty, Vijayan K.K. (2012). Aquaculture related invasion of the exotic *Artemia franciscana* and displacement of the autochthonous *Artemia* populations from the hypersaline habitats of India. Hydrobiologia, 684(1): 129-142.

Walsche C.D., Munuswamy N., Dumont H.J. (1991). Structural differences between the cyst walls of *Streptocephalus dichotomus* (Baird), *S. towicornis* (Waga), and *Thamnocephalus platyurus* (Packard) (Crustacea: Anostraca), and a comparison with other genera and species. Hydrobiologia, 212(1): 195-202

Wang S., Sun S. (2007). Comparative observations on the cyst shells of seven *Artemia* strains from China. Microscopic Research and Techniques, 70(8): 663–670.

Wurdak E.S., Gilbert J.J., Jagels R. (1978). Fine structure of the resting eggs of the rotifers *Branchionus calyciflorus* and *Asplanchna sieboldi*. Transactions of the American Microscopical Society, 97(1): 49-72.