Ultra structure of subitaneous and diapausing eggs of planktonic copepod Sinodiapitomus (Rhiniadipitomus) indicus

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Sinodiapitomus (Rhiniadipitomus) indicus produces subitaneous eggs in normal condition and diapausing eggs during unfavourable environmental condition. Diapausing eggs occurred both in the sediment samples from wild and laboratory culture system. The structure of subitaneous and diapausing eggs of the freshwater diaptomid S. (R.) indicus is described. Scanning electron microscopic (SEM) observations of these eggs showed marked variation in the surface ornamentation. Subitaneous egg has a single type of process, while diapausing egg has two different types of processes on its surface. Transmission electron microscopic (TEM) studies revealed that the plasma membrane of subitaneous egg is surrounded by a thin three-layered outer chorion, compared to highly complex, thick and four-layered outer chorion of the diapausing egg. Variation is also observed with regard to the nature of electron density of its layers.

Production of diapausing eggs is a common trait of many species of marine copepods1 and small temporary lake copepods2,3. Freshwater planktonic copepods of the family Diaptomidae are also known for the production of diapausing eggs4. The temporary lake copepods produce diapausing eggs in spring, apparently to avoid summer dry period, whereas in permanent water bodies diapausing eggs are produced to avoid summer predation by fish5,6. Distinction of diapausing egg has been an ever-persisting problem. In many instances it has been difficult to distinguish diapausing egg from subitaneous egg under light microscope, as in the case of Agaladiapitomus leptopus8, Onychadipitomus sanguineus10 and Loptadiapitomus minutus10. However, a certain degree of identification is possible by observing the morphological features such as egg colour of Agaladiapitomus clavipes11 and histological structure of egg chorion as in Hemidiapitomus ingens17 and Anomalocera patersoni13. It is only by the electron microscope, the nature of the chorion of subitaneous and diapausing eggs can be distinguished reliably from each other14.

Morphological studies on calanoid eggs have been carried out on marine species and there is very little information on the egg morphology of freshwater copepod except for the studies on diaptomids14,15 and centropods.16 In spite of rich biodiversity of the diaptomids in freshwater bodies of the Indian subcontinent, diapausing eggs have not been reported for any of the species of this group. In this paper, morphology of subitaneous and diapausing eggs of S. (R.) indicus, with special reference to their surface ornamentation and nature of the egg membrane is reported based on SEM and TEM observations.

Multiple generations of S. (R.) indicus were reared in the laboratory by feeding with Baker’s yeast solution (Saccharomyces sp.) and Chlorella sp.17. In this culture a fraction of the population switched on to the production of diapausing eggs, which were freely released into the medium and sunken to the bottom. The bottom sediment (debris) of the culture tank was siphoned out on alternate days. The eggs from the sediment were separated following the method as described by Chen and Marcus18. The sediment samples were suspended in 20 μm filtered water, sonicated for 30 min and filtered through 120 and 50 μm screen (Bolting silk cloth). The eggs and debris retained in 50 μm screen were resuspended in concentrated solution of sucrose (1 : 1, sucrose : distilled water) and centrifuged for 5 min. The material remaining in suspension was again filtered through 50 μm screen and the content was washed thoroughly and transferred to filtered water. Some of the eggs from this were transferred to a glass slide using fine pipette and observed under a compound microscope to study the morphological attributes and egg size. Occurrence of diapausing eggs in fish pond, Chetput hydrobiological station, was also investigated by obtaining sediment core (2 cm diameter, 10 cm length). Eggs from the core were also separated using the procedure mentioned above. Subitaneous eggs were collected from ovigerous female two hours after spawning. The ovisac was removed and eggs were isolated after cut opening the ovisac with the help of fine pair of needles.

The eggs that were separated from the sediment were distinguished from the subitaneous eggs based on diameter and colour. Further, eggs were maintained in the same medium (from which the eggs were collected), eggs that had neither hatched nor had begun to decay within two weeks were designated as diapausing egg. This method is more than 95% efficient in distinguishing egg types19. Generally freshwater diaptomids carry the eggs in ovisac (which is attached to the genital segment) until they hatched into free-swimming nauplii. However, eggs are also released directly in the medium (free spawned lose eggs in the sediment), which are found to be diapausing eggs. To test their viability and hatching percentage, these eggs (both collected from Chetput pond and laboratory) were washed and incubated in 100 ml rainwater with continuous aeration and hatching success was observed. Simultaneously, identical batches of eggs were maintained in the same medium (from where the eggs were collected) without aeration, to test their survival.

For light microscopic (LM) studies, eggs were either directly studied or counter stained with haematoxylin and...
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eosin. For SEM studies freshly collected eggs were washed in distilled water and fixed in 2.5% glutaraldehyde solution at 4°C for 48 h and then in 1 M phosphate buffer solution, osmicated in 1% osmium tetroxide for 2 h and dehydrated in ascending series of ethyl alcohol. The specimens were further treated with propylene oxide, air-dried below the room temperature and coated with palladium gold to a thickness of 200 Å in ion sputtering device (JEOL JFC). The specimens were scanned under a scanning electron microscope (Philips 501B Model PSEM) at 15 kV. For TEM studies, propylene oxide-treated materials were embedded in epoxy resin and ultra thin sections of 60 to 90 nm were taken using ultra microtome (ULTRA CUT JEOL). Sections were double stained in lead citrate and uranyl acetate, and studied under a transmission electron microscope (PHILIPS CM 10).

The important differences in the characteristics of subitaneous and diapausing eggs of *S. (R.) indicus* are presented in Table 1. The outer surface of subitaneous egg has small process (minute folds) with distinct space between

<table>
<thead>
<tr>
<th>Characters</th>
<th>Subitaneous egg</th>
<th>Diapausing egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>Transparent</td>
<td>Whitish</td>
</tr>
<tr>
<td>Diameter</td>
<td>108 μM</td>
<td>90 μM</td>
</tr>
<tr>
<td>Surface ornamentation</td>
<td>Single type of processes</td>
<td>Two type of processes</td>
</tr>
<tr>
<td>Fertilization membrane</td>
<td>Thin</td>
<td>Very thick</td>
</tr>
<tr>
<td>Chorion</td>
<td>3 layers</td>
<td>4 layers</td>
</tr>
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**Table 1. Differences in the structure of subitaneous and diapausing eggs of *S. (R.) indicus***

**Figure 1 a–f.**  
*a*, SEM of *Sinodiaptomus (Rhinodiaptomus) indicus* subitaneous egg (bar = 20 μm);  
*b*, Surface ornamentation of subitaneous egg (bar = 5 μm);  
*c*, TEM of the subitaneous egg chorion showing three layers L1-L3 (bar = 5 μm);  
*d*, SEM of diapausing egg (bar = 10 μm);  
*e*, Surface ornamentation of diapausing egg showing two types of processes P1 & P2 (bar = 5 μm);  
*f*, TEM of the diapausing egg chorion showing four layers L1–L4 (bar = 5 μm).
them (Figure 1a, b). The outer and inner layers of chorion of these eggs are dense, while the middle layer is less electron dense in nature (Figure 1c).

The first type of process on the diapausing egg is broad and plate-like (Figure 1d), while the second type are thin and blister-like processes which occur over the first type process (Figure 1e). Of the four layers (L1–L4) of diapausing eggs (Figure 1f), the outer and innermost layers are electron dense in nature. The outer layer has a blister-like process which is a fringed structure and forms a projected periphery of the egg. The second and third are less electron-dense layers and the former layer is striated in nature.

It is observed that the early developmental stage of the diapausing egg contains cytoplasm filled with yolk material (Figure 2a). During the course of development, cleavage takes place and as a consequence of this, blastomeres are concentrated towards one pole of the egg (Figure 2b). At the other pole a vacuole is formed, which progressively develops along the periphery of the egg causing the gastrula to get condensed into a small spherical structure (Figure 2c). In this stage, development is arrested and the egg remains dormant. The diapausing eggs collected from Chetpet pond and laboratory showed 54% and 81% hatching success respectively. In many cyclopoid copepods, it is reported that the copepodid instar or even adult secretes an organic, cyst-like covering and remains inactive within under unfavourable conditions like drought and extreme cold.

Resting egg production is supposed to be a common life history strategy adopted by the calanoids for survival in unfavourable environments. The production of diapausing eggs in large and permanent inland lakes and ponds may be due to deterioration of environment seasonally without drying up or alternatively, diapausing might be a vestigial trait indicating that the historical origin of population lies in more rigorous seasonal environment. Induction of diapausing egg production may be governed by a number of factors like photoperiod, temperature, food scarcity, over-population, pH, conductivity and gradual reduction of water quality or it may be genetically programmed into the phenotype of these organisms for their sustainability in smaller water bodies.

The size of diapausing eggs of _S. (R.) indicus_ was comparatively similar to that of different species of _Acartia_ than those of _Epiballidocera amphitrites_ and _Tortanus discicaudatus_, _Calanus sinicus_ and _Pontella mediterranea_. The surface attributes of subitaneous and diapausing eggs of _S. (R.) indicus_ do not reveal marked variation under LM examination. SEM examination shows considerable topographical variation. Such variations in the surface attributes have also been reported in the freshwater calanoids _Diaptomus sanguineus_, _Eurytemora affinis_, in marine calanoids _Anomalacera pattersoni_ and _Centropages velificatus_.

The outer layer of subitaneous eggs although reveals the presence of small processes, its surface is much smoother compared to diapausing eggs. Many calanoids, in which eggs are laid in the ovisac are known to possess smooth surface. However, the presence of smooth and spiny surfaces among diapausing eggs has been reported in many copepods. It appears that development of egg envelopes and surface ornamentation in _S. (R.) indicus_ follows closely the pattern described for diapausing eggs of _Hemidaptomus ingens_.

The processes similar to the subitaneous and diapausing eggs of _S. (R.) indicus_ were reported in A and B type of eggs of _Calanus sinicus_. Compared to this type of ornamentation, the diapausing egg of _Pontella mediterranea_ has spiny ornamentation. Apart from the surface

Figure 2a–c. LM of diapausing egg in (a) cleavage stage (bar = 10 μm); (b) early gastrula stage (bar = 10 μm); (c) gastrula stage (bar = 10 μm).
ornamentation, considerable variations were also observed with regard to the thickness of different layers of subita-
neous and diapausing eggs of S. (R.) indicus. The structure of fertilization membrane in S. (R.) indicus resembles that of Calanus sinicus[1]. However, this structure differs from that of many other calanoids[1,2,26]. The role of surface
ornamentation of subitaneous egg is not clear. The surface
ornamentation of the diapausing eggs may be an adaptive
strategy[21,26], to overcome adverse environmental conditions
and may prolong the viability of the egg and help in the
rejuvenating of the species, once suitable conditions are
restored. Further, the diapausing eggs possess a thick chitin-
ous chorion and blisters. The chitino such chorion may per-
mit the egg survive from both desiccation and passage
through the gut of predators[14]. The blisters may be con-
cerned with anti-predation. The diapausing egg of S. (R.)
indicus might be kept dormant by an endogenous factor,
which may be inherited from the female[15]. However, hatch-
ing of dormant eggs in fresh and aerated water suggests
that the exogenous factors may also serve as vital cues for
the induction of hatching of the dormant eggs. Further stu-
ries on the factor governing diapausing egg production and
their hatching induction would be useful for under-
standing the life history strategies and mass culture of
this species for their use as live feed in aquaculture.

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