ABSTRACT

Embryonic development features at difference timescales of Thai fairy shrimp, *Branchinella thailandensis*, were obtained from laboratory culture under the optimum conditions; hydration at room temperature and continuous illumination. The results indicated that the complete development of nauplius occurred within 6-8 h after hydration. As the scanning electron micrographs revealed that cysts of Thai fairy shrimp showing spherical shape with a regular pattern of polygons and three layers of the egg shell from the innermost: embryonic cuticle, alveolar layer, and outer cortex, respectively. In this study, four levels of sodium hypochlorite concentration (1%, 2%, 4% and 6%) were conducted to decapsulate cyst and compared to whole cyst. Percentage hatchability of 2% decapsulated cyst is no significance difference from whole cyst, but clearly to investigate the developmental features.

Key words: Thai fairy shrimp, *Branchinella thailandensis*, Decapsulation, Embryonic development

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INTRODUCTION

Fairy shrimps are microcrustaceans which belong to Order Anostraca. Anostracans are characterized by elongated bodies, paired compound eyes on stalks, absence of carapace, upside-down swimming motion in a metachronal rhythm and all similar appendages, which use for both swimming and feeding. Anostracans are filter feeding and sometimes scrap organic materials on solid surface (Pennak, 1989). Three species of Thai fairy shrimp (Branchinella thailandensis Sanoamuang, Saengphan and Murugan, 2002; Streptocephalus sirindhornae Sanoamuang, Murugan, Weekers and Dumont, 2000; and Streptocephalus siamensis Sanoamuang and Saengphan, 2006) have been discovered from Thai freshwaters. B. thailandensis is classified into Family Thamnocephalidae while two species later are classified into Family Streptocephalidae. B. thailandensis generally inhabits in several temporary ponds in 11 provinces of the northeast and the central of Thailand, often co-occurring with S. sirindhornae (Sanoamuang et al., 2002).

Most of Anostracan species are oviparous and internal fertilization which produced shelled eggs including B. thailandensis (Plodsomboon et al., 2012), except for some species of Artemia can also reproduce through ovoviviparous which released nauplii (Criel, 1992). Male and female of B. thailandensis reached maturity on days 7 after hatching and had mean total length 26.2±2.6 and 27.8±2.2 mm in male and female, respectively (Dararat et al., 2011). Their eggs were known as cryptobiotic cysts or cysts which produced resistant-shelled and dormant embryo. These embryos are released from maternal females in a state of gastrula developmental arrest, called as diapause (Drinkwater and Clegg, 1991). The resting eggs of freshwater fairy shrimp usually lie at the bottom of the water body and hatch in response to suitable environmental conditions about 24 h later, thus the life cycle is synchronized with a suitable environment for growth and reproduction (Munuswamy et al., 2009).

Structural morphology of whole cyst and cross-sectioned cyst have been studied in species of several genera (Gilchrist, 1978). Plodsomboon et al. (2012) demonstrated that B. thailandensis produces typical freshwater Anostracan-sculptured eggs whose external ornamentation is similar to B. australiensis (Richters, 1876), B. dubia (Schwartz, 1917), and B. frondosa Henry, 1924 (Mura, 1992), which have spherical shape and a regular pattern of polygons. In addition, Munuswamy et al. (2009) suggested that the embryo of fairy shrimp is protected by the presence of a thick outer cortex, followed by a middle alveolar layer and inner embryonic cuticle. The morphology and ornamentation of the outer cortex is unique to each species of fairy shrimp and protects the embryo to withstand long periods of desiccation. The investigation of embryonic development will be provide to understand the developmental features at difference timescales, but whole cyst is protected by the thick outer cortex making unclear observation. Thus, the decapsulation technique is commonly used and applied for removing the chorion, which composed both of outer cortex and alveolar layer.
Recently, the study of Thai fairy shrimps has mainly focused on their taxonomic status, environmental context and cultivation of these species in order to use them as a new live food for freshwater aquatic animals such as prawns, shrimp and ornamental fish (Boonmak et al., 2007). Dararat et al. (2012) illustrated that all three species of Thai fairy shrimps contain high nutritional value that are needed for growth and reproduction, closed to Artemia sp. and providing an alternative to Artemia sp. In addition, Boonsoong and Bullangpoti (2012) used B. thailandensis nauplii in acute toxicity test of herbicides and insecticides. Therefore, our objective was to study embryonic developmental features at difference timescales from the encysted gastrula to the fully formed nauplius for supplying document data.

MATERIALS AND METHODS

The cysts of B. thailandensis were obtained from Faculty of Agriculture and Natural Resources, Rajamangala University of Technology Tawan-ok (RMUTTO), Bangpra campus, Chonburi province, Thailand. They were kept in plastic bags.

1. Scanning electron microscopy

Whole cysts and cross-sectioned cysts using a fine surgery knife were taken to study surface and internal topography, respectively. Samples were mounted on a standard metal stub, coated with gold (< 10 nm) and observed with a JEOL JSM-5600LV scanning electron microscope at a voltage of 10 kV.

2. Decapsulation and Hatching efficiency assay

The cysts of B. thailandensis were hydrated in de-chlorinated water for 30 minutes. Hydrated cysts were decapsulated using sodium hypochlorite which contain 1, 2, 4 and 6% of active ingredients (Halter bleach contain 6% of active ingredients) and stirred for 5 minutes until the decapsulation reaction was complete, that denotes as the gradual color of cysts were changed from dark brown to orange and white. Decapsulated cysts were washed thoroughly with de-chlorinated water until they were cleared of the chemicals. The assays were performed in 2x3 multi-well plates for five treatments (10 mL of de-chlorinated water) and five replicates per treatment group with continuous illumination of approximately 4,500 Lux at room temperature (25-27 °C) for hatching. Hatched eggs were counted at 6, 8 and 24 h under stereomicroscope. One-way ANOVA was used to determine an optimum concentration for percentage hatchability.

3. Time-lapse of embryonic development

Decapsulated cysts using 2% sodium hypochlorite were incubated in 10 mL of de-chlorinated water in 2x3 multi-well plates with continuous illumination of approximately 4,500 Lux at room temperature (25-27 °C) for hatching. Samples were randomly selected since 1 to 8 h and fixed in 95% ethanol. Fixed samples were immersed in distilled water for 1 minute and dropped on glass slides with two drops of 100% glycerine. Mounted slides were observed using light microscope for finding developmental embryos.
RESULTS AND DISCUSSION

1. Scanning electron microscopy

The scanning electron micrographs reveal the characteristics of cysts. Cysts of *B. thailandensis* showing spherical shape with a regular pattern of polygons. The external ornamentation is made up of the ridges with sloping sides and depression regions (Figure 1a). Diameter of cyst is between 180-200 µm. Pores and spiny projections on sculptured surface of egg shells are prominent (Figure 1b,c). Structural morphology of cross-sectioned cyst demonstrates three layers of the egg shell (Figure 1d). The innermost layer enveloping the embryo is the cuticular layer or embryonic cuticle, which appears in decapsulated cysts under light microscope. The middle is the alveolar layer with small hollows and the outermost of egg shell is defined as thick outer cortex.

![Figure 1. Scanning electron micrographs.](image)

It can be seen that the embryo of Thai fairy shrimp *B. thailandensis* is protected from the external environment by the presence of a thick outer cortex, followed by a middle alveolar layer and an innermost embryonic cuticle. According to Plodsomboon *et al.* (2012), the structure of the sculptured shell of *B. thailandensis* was reported to belong to the group without a sub-cortical space and
composed an outer cortex (~20µm), the middle smaller alveolar layer (~5µm) and the innermost cuticular layer (~1µm). The structural of egg shell supports to withstand long periods of desiccation. Otherwise, the morphology and ornamentation of egg shell is unique to each species of fairy shrimp that can be used to classify and predict the capability of fairy shrimp for inhabiting in stressful or unpredictable environments.

2. Decapsulation and Hatching efficiency assay

The comparison of percentage hatchability of *B. thailandensis* between whole cysts or non-decapsulated cysts which denoted as control group revealed that whole cysts as the highest and 6% of sodium hypochlorite decapsulated cysts treatment as the lowest percentage hatchability in all observed time. There were no significant differences (*p*>0.05) in hatching efficiency between whole cysts and decapsulated cysts using 2% sodium hypochlorite of all experiments time. The percentage of hatchability of whole cysts, 1% and 2% decapsulated treatments increased significantly (*p*<0.05) when increasing time through 6, 8 and 24 h, respectively. While 4% decapsulated cysts treatment increased significantly (*p*<0.05) at 24 h, but no significance differences (*p*>0.05) among 6 and 8 h. In case of 6% decapsulated cysts treatment, it increased significantly (*p*<0.05) between 6 and 24 h, but no significant differences (*p*>0.05) between 6 h and 8 h with 8 h and 24 h. There were no significance differences (*p*>0.05) in the percentage of hatchability between 1% and 2% decapsulated cysts treatment at 8 h, while significance differences (*p*<0.05) at 6 h. In addition, after incubation for 24 h, there were no significance difference (*p*>0.05) between 1% and 4% decapsulated cysts treatment. In case of 6% decapsulated cysts treatment, the significance showed difference (*p*<0.05) from each other. As following the results, indicated that 2% of sodium hypochlorite is the appropriate concentration which clear the chorion of cysts without affecting the viability of embryos.

The continuous observation of embryonic development of whole cyst is so difficult and unclear. Hence, the decapsulation technique is used and improved for removing the outer part of the shell or chorion without affecting the viability of the embryos (Sorgeloos *et al.*, 1977). Nearby previous study of Sorgeloos *et al.* (1977), the optimum decapsulated concentration (2% sodium hypochlorite) using in this study is suitable for investigating the embryonic developmental features. The advantages of decapsulation for the practical use in laboratory work and aquaculture are quite obvious: 1) complete separation of nauplii from the hatching debris since remained the only embryonic cuticle; 2) disinfection on external surface of cyst shells; 3) possible direct ingestion and digestion to decapsulated cyst by fish and crustacean larvae, which reduced the deleterious effects (Sorgeloos *et al.*, 1977). However, appropriateness of kind and concentration of decapsulated substances is important because it can be affecting the viability of the embryos.
3. Time-lapse of embryonic development

Embryonic development is observed ongoing hydration since 1 h to 8 h (Figure 2). The developmental process indicates the morphogenesis of developing embryo into fully formed nauplius, which is a larval form with three pairs of appendages and a single median eye. The decapsulated cyst after 30 minutes of hydration clearly shows prominent yolk granules in the developing embryo. After 1 h of incubation, the embryo is still undifferentiated and the yolk granules are gradually utilized (Figure 2a). After 2 h, yolk granules are utilized for developing, subsequently the embryo within the embryonic cuticle divides into two hemi-halves to differentiate into the anterior and posterior regions, by that the anterior part is broader than the posterior part. Furthermore, signs of morphogenesis occur as showing the development of larval lime buds (Figure 2b). After 3 h of hydration, the anterior region develops into a brown head region and posterior as the thoracic and abdominal region. In addition, the appendages will be developing (Figure 2c). After 4 h, substantially changes occur in the embryo which developing the antennae and mandibles, together with beginning to appear the median naupliar eye (Figure 2d). After 5 h, appearance of development completed to the nauplius stage, which the first and second antennae and mandibles develop as well and the anal pore can be observed, followed by hatching (Figure 2e). Since after 6 h until 8 h incubation, which is defined as the nauplius further develops and just hatches from embryonic cuticle into free swimming nauplius (Figure 2f-h)

![Figure 2. Embryonic development in Branchinella thailandensis; a, After 1 h, the embryo is still undifferentiated and the yolk granules are gradually utilized (Arrow). b, After 2 h, the embryo within the embryonic cuticle divides into two hemi-halves and showing the development of larval lime buds. c, After 3 h, the anterior develops into a brown head and posterior as the thoracic-abdominal region. d, After 4 h, substantially developing the antennae and mandibles, together with beginning to appear the median naupliar eye. e, After 5 h, complete development to nauplius, the first and second antennae and mandibles develop as well as the anal pore. f-h, After 6-8 h, the nauplius hatches from embryonic cuticle into free swimming nauplius. EC; embryonic cuticle, LB; lime bud, A; anterior part, P; posterior part, N; median naupliar eye, 1st an; 1st antennae, 2nd an; 2nd antennae, ma; mandibles, and ap: anal pore.]
Morphogenesis and embryonic development of *B. thailandensis* takes place to fully formed of nauplius occurred within 6-8 h after hydration, which faster than the complete development of *Streptocephalus dichotomus* occurred within 8-12 h (Munuswamy et al., 2009). In the first hour *B. thailandensis* and *S. dichotomus* are similar in the point of yolk granules utilization. Since after 2 h, the developmental features of *B. thailandensis* take rapid phase, which the first sign of morphogenesis is the appearance of larval appendicular buds and the second is the formation of median naupliar eye after 2 h and 4 h of hydration, respectively. According to habitat observation, which illustrated that *B. thailandensis* generally inhabits in short-lasting ponds, which have extreme fluctuations of water level and short period of inundation approximately 1-2 months. Therefore, fairy shrimp *B. thailandensis* has also developed their biological characters to help them persist under unpredictable habitats, including the hatching pattern, maturation time and life span (Vanschoenwinkel et al., 2010). Hence, this is the first reported of the embryonic development at difference timescales of Thai fairy shrimp.

**CONCLUSION**

Cysts of *B. thailandensis* show spherical shape with a regular pattern of polygons. Pores and spiny projections on sculptured surface are prominent. Structural morphology of cross-sectioned cyst reveals three layers of the egg shell (innermost embryonic cuticle layer, alveolar layer and outer cortex). The percentage of hatchability of whole cysts, 1% and 2% decapsulated treatments, increased when increasing time through 6, 8 and 24 h, respectively and no differences in hatching efficiency between whole cysts and 2% sodium hypochlorite decapsulated cysts of all experiments time. As the results, indicated that 2% of sodium hypochlorite is the optimum concentration, which clear the chorion of cysts without affecting the viability of embryos. Embryonic development of *B. thailandensis* to nauplius under optimum conditions completed within 6-8 h, which dramatic changes occurred around after 2-4 h by appearance the larval limb buds and median naupliar eye. After that, the embryo will be further developing into fully formed nauplius.

**REFERENCES**


