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THESIS

CRYPTOBIOTIC CYST STRUCTURES AND EMBRYONIC DEVELOPMENT OF TWO THAI FAIRY SHRIMPS, *Branchinella thailandensis* and *Streptocephalus sirindhornae*

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Suwannit Sukarawan 2014: Cryptobiotic Cyst Structures and Embryonic Development of Two Thai Fairy Shrimps, *Branchinella thailandensis* and *Streptocephalus sirindhornae*. Master of Science (Biology), Major Field: Biology, Department of Zoology. Thesis Advisor: Assistant Professor Boonsatien Boonsoong, Ph.D. 67 pages.

This study aimed to investigate the structures of cyst and embryonic development of two Thai fairy shrimps (*Branchinella thailandensis* and *Streptocephalus sirindhornae*). As the scanning electron micrographs revealed that cysts of *B. thailandesnsis* and *S. sirindhornae* show spherical shape with a regular pattern of polygons. Diameter of cyst is approximately 200 μm and 150-180 μm, respectively. Cyst of *B. thailandensis* appears pores and spiny projections, while spines are disappeared in *S. sirindhornae*. Structural morphology of cross-sectioned demonstrated three layers of egg shell in *B. thailandensis* (innermost embryonic cuticle, alveolar layer and outer cortex), indistinct three layers in *S. sirindhornae* (innermost embryonic cuticle, alveolar layer which is not demarcated with inner layer and outer cortex). In this study, four levels of sodium hypochorite concentration (1%, 2%, 4% and 6%) were conducted to decapsulate cyst and compared to whole cyst. Percentage hatchability of 2% NaOCl decapsulated cyst is no significance difference from whole cyst, but clearly to investigate the developmental features. Embryonic development at difference timescales of *B. thailandensis* and *S. sirindhornae* were obtained from laboratory culture under the optimum conditions; hydration at room temperature and continuous illumination. The results indicated that the complete development of nauplii occurred within 5 and 7 hours after hydration, respectively. *B. thailandensis* shows slightly rapid development more than *S. srindhornae*. The preliminary study on the occurrence of stress proteins, p26 and artemin, by observing the bands which appear nearly 26 kDa, which are the critical components of cryptobiotic cysts to survive under stress environments.

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LIST OF ABBREVIATIONS

^{o}C	=	degree Celcius
CO_2	=	carbon dioxide
g	=	round per minute
kDa	=	kilodalton
kV	=	kilovolt
h	=	hour
m	=	meter
mA	=	milli-ampere
mg g^{-1}	=	milligram per gram
ml	=	milliliter
µg g^{-1}	=	microgram per gram
µl	=	microliter
nm	=	nanometer
PBS	=	phosphate buffered saline
ppt	=	part per thousand
V	=	Volt

CRYPTOBIOTIC CYST STRUCTURES AND EMBRYONIC DEVELOPMENT OF TWO THAI FAIRY SHRIMPS, *Branchinella thailandensis* and *Streptocephalus sirindhornae*

INTRODUCTION

Fairy shrimps are microcrustaceans which belong to Order Anostraca. Anostracans are characterized by elongated bodies, paired compound eyes on stalks, absence of carapace, constantly swim on their backs in the water column and have all similar thoracic appendages, which use for both swimming and feeding. Anostracans are filter feeding and sometimes scrap organic materials on solid surfaces (Pennak, 1989). The distribution of fairy shrimps through all types spatial of the world include 26 genera and around 300 species, which mostly distribute and inhabit in temperate zone (Belk and Brtek, 1995, 1997; Dumont and Negrea, 2002). They are usually appear in temporary ponds or ephemeral habitats. Three species of Thai fairy shrimps (*Streptocephalus sirindhornae* Sanoamuang, Murugan, Weekers and Dumont, 2000; *Branchinella thailandensis* Sanoamuang, Saengphan and Murugan, 2002 and *Streptocephalus siamensis* Sanoamuang and Saengphan, 2006) have been discovered from Thai freshwaters. *B. thailandensis* is classified into family Thamnocephalidae, while two species of genus *Streptocephalus* belong to 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*, which more widely distributes (Sanoamuang *et al*., 2002). Almost of anostracan species are internal fertilization and oviparous, which produce shelled eggs including all three species of Thai fairy shrimps (Plodsomboon *et al*., 2012). Eggs of fairy shrimps are known as cryptobiotic cysts or cysts, which are released from maternal females in a state of gastrula developmental arrest, called as diapause and confined by resistant-shelled (Drinkwater and Clegg, 1991). The resting eggs of freshwater fairy shrimp usually lie at the bottom of the water body and hatch in the suitable environmental conditions around 24 hours, thus the life cycle is synchronized with a suitable environment for growth and reproduction (Munuswamy *et al*., 2009). Structural morphology of cyst and cross-sectioned cyst have been studied in species

of several genera (Gilchrist, 1978). Dararat *et al*. (2012) demonstrated that *B. thailandensis* produces typical freshwater anostracan sculptured eggs, which appear spherical shape with a regular pattern of polygons and also show prominent pores on surface. Whereas *S. sirindhornae* produces sculptured eggs, which are smaller than cysts of *B. thailandensis* but they are slightly different in the structure and ornamentation. 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 surface topography of the outer cortex is unique to each species of fairy shrimp and protects the embryo to withstand long periods of desiccation, which can be used to classify and predict the capability of fairy shrimp for inhabiting in stressful or unpredictable environments. Interestingly, there are several specialized biochemical adaptations including synthesize and accumulate biomolecules like glycerol, trehalose sugar and stress proteins. Previous studies have been reported the occurrence of stress proteins (p26 and artemin), which are thermotolerance or heat shock proteins in *Artemia*. Researches on the stress proteins (p26 and artemin) have been increased greatly in recent year for investigating the occurrence of this proteins outside the genus *Artemia*. Munuswamy *et al*. (2009) reported that p26 and artemin were found in cysts of *Streptocephalus dichotomus*, unlike some species of fairy shrimp (*Branchinecta* spp.) or other related anostracans. Recent studies of Thai fairy shrimps have mainly focused on their taxonomic categories, environmental context and cultivation of these species in order to use them as a new live food for freshwater aquatic animals (Boonmak *et al*., 2007). In addition, Boonsoong and Bullangpoti (2012) use *B. thailandensis* nauplii in acute toxicity test of herbicides and insecticides. Nowadays, the investigation of embryonic development will be providing to understand the developmental features at difference timescales.

Therefore, our objectives were to study embryonic developmental features at difference timescales of two Thai fairy shrimps *B. thailandensis* and *S. sirindhornae* from the encysted gastrula to the fully formed nauplius of decapsulated cyst for selecting the appropriate time of the developmental stage, which can be applying to further studies. Furthermore, this is the first time of the study on the embryonic development at difference timescales in the Thai fairy shrimps.

OBJECTIVES

1 . Investigate the structures of cryptobiotic cysts of two Thai fairy shrimps *Branchinella thailandensis* and *Streptocephalus sirindhornae.*

2. Study embryonic developmental features at different timescales of two Thai fairy shrimps *Branchinella thailandensis* and *Streptocephalus sirindhornae* from the cryptobiotic cyst to the fully formed nauplius.

LITERATURE REVIEW

1. Biology

Fairy shrimps are microcrustaceans, which belong to order Anostraca. Anostracans are characterized by elongated bodies, paired compound eyes on stalks, absence of carapace, constantly swim on their backs in the water column, and have all similar thoracic appendages (phyllopodia), which use for both swimming and feeding. Almost of fairy shrimps are range in size between one centimeter to three centimeters in length (Dumont and Munuswamy, 1997; Dumont, 2004). Except for some species *Branchinecta gigas*, which grows up to ten centimeters in adult body length (Dumont, 2004). The body of fairy shrimp is distinctly separated into three parts; head, thorax and abdomen. The head consists of a pair of compound eyes on prominent stalks, unpaired median naupliar eye, two pairs of antennae, and the jaws (mandibles). The first pair of antennae are known as antennules, which are usually small, unsegmented and uniramous. The second pair are long and cylindrical in females, but they are enlarge and special for holding the female during copulation in males. In some groups, males have an additional frontal appendages for clasping females. The mandibles are strong and modify to a hood-like shaped which located upper lip for hindering the flow of water towards the mouth. The thorax of most fairy shrimps consist of 11 segments and a few have 17-19 thoracic segments. Every segment bears a pair of leaf-like appendages or phyllopodia that are similar in structure. Every appendage has two or three lobes on the outer side, which contains the breathing organs, and one lobe for padding. There are six lobes on the inner side for pushing the water to the mouth opening. On the ventral side of the body is a deep groove inside for water flowing to the mouth. The abdomen is cylindrical and consists of about eight segments without appendages, and appears a telson plate, which bears two flattened caudal rami or cercopods (Dumont and Munuswamy, 1997; Dumont, 2004).

Fairy shrimps are filter feeding, which filter food indiscriminately from water while they are swimming, and also scrap algae and other organic materials from solid surfaces. Overall, the diet of fairy shrimps consist of algae, bacteria, protozoa, rotifers and organic detritus (Pennak, 1989).

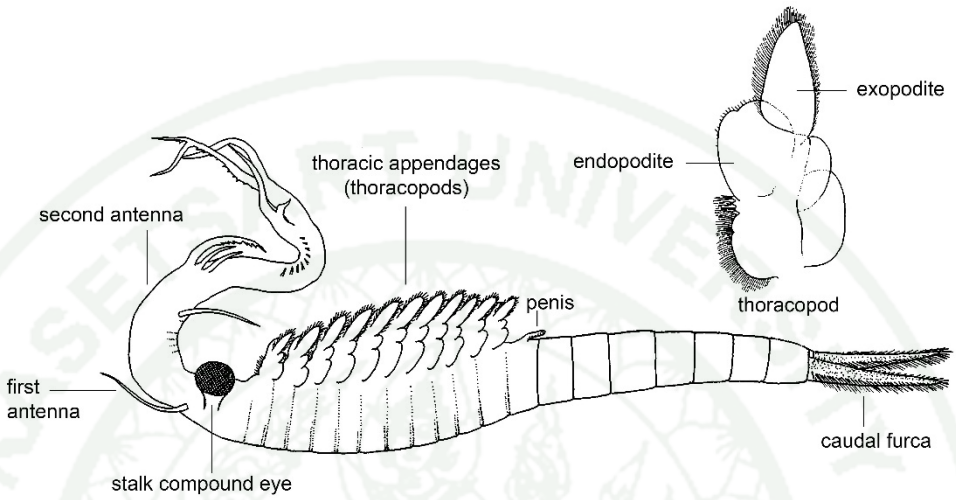

Figure 1 Morphology of male fairy shrimp, *Branchinella thailandensis*.

Source: Boonsoong (2014)

2. Ecology and Distribution

Anostracans inhabit in inland waters ranging from hypersaline lakes to lakes that are almost devoid of dissolved substances. They are also archetypal crustaceans in ephemeral waters. The relatively large size of fairy shrimp together with slow locomotion make them an easy target for predator fish and waterfowl, thus their distribution will be restricted to extreme environments with fewer predators. Fairy shrimps are fascinating animals that appear in temporary pools such as roadside ponds, rice field ditches and grassy vernal pools (Gilchrist, 1978). Degree of rainfall and seasonal flooded depression are important factors for determining the water level in these temporary habitats, which can be divided into different types of habitat including predictable and unpredictable ponds. Under brief of temporary water inundation or unpredictable habitats, most aquatic animals must adapt their life history traits to survive under this environmental stressful condition by having rapid growth,

early egg hatching and shorter life spans than those in predictable habitats (Hildrew, 1985). Fairy shrimp is one of the anostracan group that has adopted their life history including early hatching, rapid maturation and early start of egg production to effectively inhabit in temporary ponds (Dararat *et al*., 2011). Their eggs or cysts are produced resistant-shelled and dormant embryo and they are usually lie at the bottom of the water body and hatch in response to suitable environmental conditions about 24 hours later, thus the life cycle is synchronized with a suitable environment for growth and reproduction (Munuswamy *et al*., 2009).

In term of distribution, fairy shrimps distribute in a relation to the movement of eggs by wind (Graham and Wirth, 2008). Among of the three species of Thai fairy shrimps, *S. sirindhornae* is the most widely distributed species in Thailand (Sanoamuang *et al*., 2000), which is in agreement with the findings of Brtek and Mura (2000) who reported that *Streptocephalus* is the most widespread and tolerant genus of fairy shrimps in the world. The widespread distribution of *S. sirindhornae* in Thailand may depends on their sexual ability, which can be produced a lot of eggs, up to 17,865 eggs per individual female. Therefore, their eggs would have more chance to be dispersed by wind than the other two species. Another revision could be explained that *S. sirindhornae* seems to be tolerant species in Thailand. It occurs in a wide range of habitats with many environmental variables when compared with *B. thailandensis* (Sanoamuang *et al.,* 2000). *B. thailandensis* appears to be restricted to the central and northeast part of Thailand, which has been collected from 11 provinces; Lop Buri, Chainat, Kanchanaburi, Ratchaburi, Suphanburi, Uthai- Thani, Khon Kaen, Roi Et, Mahasarakham, Nakhon Ratchasima, and Chaiyaphum provinces (Sanoamuang *et al.*, 2002). Whereas, *S. siamensis* which is the latest newly species of Thai fairy shrimp. They are rare species and have been found in Suphanburi and Kanchanaburi provinces, central of Thailand (Sanoamuang and Saengphan, 2006).

3. Taxonomy

In the tropical monsoon region of Southeast Asia, only three species have been recorded; *Streptocephalus dichotomus* Baird, 1860 from Myanmar (Belk and Esparza, 1995), *Streptocephalus javanenensis* Brehm, 1995 from Indonesia (Brehm, 1995, Belk and Brtek, 1995) and *Streptocephalus sirindhornae* Sanoamuang, Murugan, Weeker and Dumont, 2000 from Thailand (Sanoamuang *et al*., 2000). Hence, anostracans have been continuous discovering. Three species of Thai fairy shrimps have been reported from Thailand. The first discovered species and was described from temporary pond in Khon Kaen is *S. sirindhornae*, which is previously called as freshwater *Artemia* by local researchers (Sanoamuang *et al*., 2000). Two years later, the second species, *Branchinella thailandensis* Sanoamuang, Saengphan and Murugan 2000, was discovered from several localities in 11 provinces in the central and northeast region of Thailand. The latest newly species of Thai fairy shrimp has been discovered six years later is *Streptocephalus siamensis* Sanoamuang and Saengphan, 2006, which is rare species, found only five temporary localities in Kanchanaburi and Suphanburi provinces, central of Thailand. This important discovery will provide to understand the taxonomic status of anostracans because this also represent the members of fairy shrimps in Thailand and the first recorded of the family Thamnocephalidae from Southeast Asia.

4. Reproduction

The major reproductive mode of anostracan species is oviparous, which produced resistant-shelled embryos or cysts, except for some species of *Artemia* can also reproduce through ovoviviparous, which released nauplii (Browne, 1980; Criel, 1992). As a typical freshwater fairy shrimp species, *B. thailandensis* exhibits oviparous and internal fertilization (Sanoamuang *et al*., 2002) as species of the genera *Branchinecta*, *Dendrocephalus*, *Streprocephalus* and *Thamnocephalus* (Roger, 2002). There are generally marked differences reproductive structures between male and female. Males have modified second paired of antennae for clasping and holding the female during mating and males also have penes (Dumont and Munuswamy, 1997; Dumont, 2004). Anostracan females do not store sperm, thus copulation is required

to fertilize with each oocyte clutch (Bowen, 1962; Prophet, 1963; Munuswamy and Subramonium, 1985). Plodsomboon *et al*. (2012) found that *B. thailandensis* females receive the copulation after molting and ovulation. Generally, receptive anostracan females have an empty ovisac and ripe oocytes in the lateral pouches (Bowen, 1962; Metalli and Ballardin, 1972; Belk, 1991). Plodsomboon *et al*. (2012) reported that two reproductive patterns are observed within anostracans. The first one, oocytes pass to the ovisac only after copulation, that is not controlled by an endogenous egg cycle, including *Branchinecta*, *Branchipus* and *Eubranchipus* (Belk, 1991; Maeda-Martínez *et al*., 2003), and in the other, that is controlled by an endogenous egg cycle, which found in *B. thailandensis* including species of *Artemia*, *Tanymasti*, *Streptocephalus* and *Thamnocephalus* (Bowen, 1962; Garreau de Loubresse, 1974; Murugan *et al*., 1996; Maeda-Martínez *et al*., 2003)

Reproductive cycles in oviparous species are productive when copulation occurs and oocytes are fertilized inside the ovisac and later coated with shelled-gland secretions to form eggs, while in the ovoviviparous mode in some species of *Artemia*, fertilized oocytes are not coated and continuously developed to the nauplius stage (Criel, 1980, 1992; Maeda-Martínez *et al*., 2003). Plodsomboon *et al*. (2012) also found no oocyte resorption in unmated *B. thailandensis* females; the oocytes remained unaltered or eventually disintegrated in the ovisac to form a milk-like substance. The unaltered oocyte and the milk-like substance were later expulsed from the ovisac in the same manner as females releasing fertilized eggs. The copulation of *B. thailandensis* appears as the same mechanism of some streptocephalids, which is required to trigger shell formation (Murugan *et al*., 1996).

After mating for two to three days, the fertilized eggs are released and dumped into the water when they start developing. Eggs of some species may sink to the bottom, while eggs of other species may float to the surface and drift to the lake shore to be deposited. Females can produce two types of eggs; thin shelled summer eggs, and thick shelled winter eggs. The summer eggs will hatch rapidly and populate in the pool during the same season they are laid. Whereas, the winter eggs remain in the mud at the base of the pool and dry out in the pool. The eggs have been subjected

the experience to temperatures of as high as 99°C and as low as -190°C while usually remained viable varies between six to ten months. Winter eggs usually hatch in the spring or about 30 hours after being exposed to water (Dumont, 2004).

Overall, females may lay up to forty broods of egg in lifetime, with each brood or clutch containing several hundred eggs, yielding a total fertility of up to 4,000 eggs per female (Dumont, 2004). Boonmak *et al.* (2007) reported that Thai fairy shrimps are substantially fecundity when compared to other previously studied species. Brood frequencies of *S.sirindhornae* and *B. thailandensis* were 1.27±0.07 and 1.14±0.04 days, respectively. The numbers of broods and cysts per female of *S. sirindhornae* and *B. thailandensis* were 37.62±2.29 and 14.20±4.60 broods and 18,685±2,130 and 6,699± 2,094 cysts, respectively. Comparative data indicated that *S. sirindhornae* has a longer life span (64.50±6.81 days) than *B. thailandensis* (25.40±9.03 days). It is suggested that *S. sirindhornae* produces more cysts than *B. thailandensis* and suitable for the commercial production of eggs, while *B. thailandensis* is appropriate for mass production and serve as live food for aquatic animals.

The egg of fairy shrimp is known as cryptobiotic cyst or cyst, which produce resistant-shelled and dormant embryo. These egg is released from maternal female in a state of gastrula developmental arrest, is called as diapause (Drinkwater and Clegg, 1991) which is characterized by interruption of development and extremely reduced metabolism (Drinkwater and Crowe, 1987; Drinkwater and Clegg, 1991; Clegg, 1997). The morphology and ornamentation of the egg shell is unique to each species of fairy shrimp and protects the embryo to withstand for long periods of desiccation (Munuswamy *et al.*, 2009). Structural morphology of whole cysts and cross-sectioned cysts have been studied in species of several genera (Gilchrist, 1978; De Walsche *et al.*, 1991; Maeda-Martínez *et al.*, 1992; Hill and Shepard, 1997). Generally, cyst shell is composed of three layers; an outer cortex, followed by a middle alveolar layer and an inner embryonic cuticle. The composition of outer cortex and middle alveolar layer is the external layer of cyst that is known as chorion, which appears hard and dark brown (Sorgeloos *et al.*, 1977). Scanning electron microscopy revealed that the external layer of most species were separated by a subcortical space, which

intercommunicates with the meshwork of the alveolar layer (Gilchrist, 1978). Obregón-Barboza *et al*. (2002) studied in *Branchinecta*, and reported that three types of shell; 1) shells with a subcortical space present, the outer cortex and inner alveolar layer completely separated from each other; 2) shells with a subcortical space present, but the outer cortex and inner alveolar layer are not completely separated from each other; and 3) shells with a subcortical space absent and composed of a single spongy cortex. De Walsche *et al*. (1991) illustrated that the surface topography and layer of the cyst wall of fairy shrimps *Streptocephalus dichotomus, Streptocephalus torvicornis* and *Thamnocephalus platyurus* based on scanning electron micrographs, shown that cyst walls are not invariably bilayer, but may be composed of up to four layers. Hence, the structural morphology of species specific in all taxa will be further studying. However, cyst wall structure within single cyst may differs according to the topographical location on the cyst. Both of *B. thailandensis* and *S. sirindhornae* produce typical freshwater anostracan-sculptured eggs. The external ornamentation of *B. thailandensis* is similar to *Branchinella australiensis* (Richters, 1876), *Branchinella dubia* (Schwartz, 1917), and *Branchinella frondosa* (Henry, 1924) which appear spherical shape and a regular pattern of polygons and also exposes prominent pores on surface (Plodsomboon *et al*., 2012). While, external ornamentation of *S. dichotomus* shows spherical shape and a regular pattern of polygons including prominent pores on surface (Munuswamy *et al*., 2009).

5. Embryonic development and Hatching efficiency

Cryptobiotic cysts of anostracans are in a state of developmental arrest and anoxic, subsequently undergo to a reduction in metabolism, which corresponds to survive severe in desiccation and stressful conditions. Thus, the life cycle is synchronized with a favorable environment for hatch, growth and reproduction. Hall and MacDonald (1975) demonstrated that the osmotic processes and the possible role of glycerol for hatching mechanism in anostracan, *Chirocephalus diaphanus* Prévost. In this mechanism, glycerol is thought to accumulate in the egg as a result of the metabolism of the embryo, subsequently increased osmotic pressure in cyst and caused to water enter to the cyst until the hydrostatic pressure is sufficient to cause rupture the egg

shell. It is recognized that, the hatching in anostracans are two staged phenomenons, first is a process of "breaking" which glycerol is involved, and preceding true "hatching" which glycerol is not involved. There are several necessity factors that involve hatching mechanism of fairy shrimp; hydration, osmotic pressure, dissolved oxygen, temperature, pH, and light, unlike salinity is essential for brine shrimp (Saengphan *et al*., 2005). Murugan and Dumont (1995) illustrated that the influence of light on the hatchability of *Thamnocephalus platyurus* Packard cysts that a maximum of 65±6% of hatchability was recorded within seven days at 2,500 Lux continuous light regime. Hatching was at a minimum during the first two days, peaked between the third and fourth days, and decreased thereafter. Hatching success was a function of duration of light exposure. Eight percent of cysts hatched in the dark, while cysts exposed to 24 hours light and subsequently incubated in the dark showed 27±2% hatching. Besides, the optimum standard conditions for hatching efficiency, there are the other factors that influence on egg hatch, such as artificial accelerators and inhibitors. Dumont *et al*. (1992) examined cyst hatching of anostracan species *T. platyurus* and *S. dichotomus*, which were significantly accelerated but not increased in term of number by applying the morphogen retinoic acid (RA) and almost quantitatively inhibited by the Calcium-channel blockers Nifedipin and Verapamil. Furthermore, parental rearing condition is influence to egg fecundity and hatching efficiency. Mura *et al*. (1999) demonstrated that the influence of rearing conditions on cyst production and hatching in the fairy shrimp *Chirocephalus ruffoi* and evaluated by freshly hatched nauplii under two different conditions; in indoor culture at constant temperature and photoperiod, and in outdoor culture under naturally fluctuating temperature and varying photoperiod. Percentage of hatchability was minimum for those laid under outdoor fluctuating conditions (2%) whereas, cysts produced at constant conditions showed a much higher hatching percentage (46.7%).

For the cyst hatching pattern of Thai fairy shrimps, Saengphan *et al*. (2005) have been reported the influence factors on the hatching of fairy shrimp *B. thailandensis* are also related to brood order and response of wet and dry periods. This study showed that a wet period has strong influence on the cyst hatching of *B. thailandensis*, and cysts of the first brood hatched less successfully than those of sixth and eleventh broods.

As the results, indicated that the cysts required a period of retention in the parental medium for 2-4 weeks to complete their embryonic development before hatching. The early brood required a longer wet period than the late broods. In contrast, the hatching behavior of many other species, drying is not absolutely essential for cyst hatching. Hence, anostracan species including fairy shrimps have the ability to survive reversible dehydration during one or more stages of their life history, which is known as anhydrobiosis (Munuswamy *et al*., 2009).

Saengphan *et al*. (2005) suggested that cysts of *B. thailandensis* hatching mostly took place within 24 hours after incubation. Dararat *et al*. (2011) examined the daily observation on egg hatching of three Thai fairy shrimps for seven days after incubation. The results revealed that *B. thailandensis* hatched mostly within the first three days, which represented hatchability of 75.66% on the first day, followed by 8.66% on the second day, and 3.33% on the third day. Whereas, *S. sirindhornae* and *S. siamensis* hatched within four days, which had a lower hatching rate than *B. thailandensis*. Eggs of *S. sirindhornae* and *S. siamensis* hatched 45.0 and 18.0% on the first day, followed by 11.33 and 21.66% on the second day, 7.0 and 7.66% on the third day, and 0.66 and 3.33% on the fourth day, respectively. As the observed data, indicated that *B. thailandensis* has the shorter hatching time, while *S. sirindhornae* and *S. siamensis* have the longer hatching time.

Munuswamy *et al*. (2009) investigated the complete morphogenesis and embryonic development of fairy shrimp *S. dichotomus,* which occurs within 8-12 hours after hydration. The decapsulated cysts after 1 and 2 hours of hydration show prominent yolk granules and still undifferentiated embryos, but gradually yolk granules are utilized. Signs of morphogenesis occur after 3 hours of hydration, which embryos divide into two hemi-halve; one half develops into the anterior region and the other develops into the posterior region. After 4 hours of incubation, the anterior region develops into brown head and the posterior region as the abdominal region. Dramatic changes occur in the embryo around after 5 hours of hydration, which appear the first and second antennae and mandibles, and the median naupliar eye can be observed prominently. After 7 hours, the nauplius further develops inside the embryonic cuticle.

After 8 hours of incubation, the embryo emerges from the cyst and the fully formed nauplius is released into the medium. It can be seen that morphogenesis and embryonic development of fairy shrimp *S. dichotomus* takes place in a rapid phase and the whole nauplius is ready to hatch in about 8 hours after hydration. This rapid development and fast hatching response is an adaptation to the stressful temporary pool inhabitants.

6. Cultivation and Nutritional efficacy

Since three species of Thai fairy shrimps have been discovered in Thailand, thus the study of Thai fairy shrimps has mainly focused on their life history (Boonmak *et al*., 2007; Dararat *et al*., 2011), the optimal methods for egg hatching (Saengphan *et al*., 2005), black disease (Saejung *et al*., 2011), and acute toxicity test of herbicides and insecticides (Boonsoong and Bullangpoti, 2012). Attempts have been made to culture these species in order to use them as a new live food source for freshwater aquatic animals such as prawns, shrimp and ornamental fish (Saengphan *et al*., 2005; Sanoamuang, 2005; Sanoamuang and Saengphan, 2006; Boonmak *et al*., 2007; Plodsomboon and Sanoamuang, 2007; Sriputhorn and Sanoamuang, 2007; Saengphan and Sanoamuang, 2009; Dararat *et al*., 2012). 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 can be providing for an alternative to *Artemia* sp. The nutritional analysis revealed that *S. sirindhornae* contains higher crude protein (74.41%) than *B. thailandensis* (64.65%) and *S. siamensis* (50.24%), compared to *Artemia* sp. (56.25%). The highest amino acid content is recorded in *S. sirindhornae* (784.92 mg g^{-1} dry weight) followed by that of *B. thailandensis* (596.12 mg g^{-1} dry weight) and *S. siamensis* (439.58 mg g^{-1} dry weight). In term of essential amino acid (EAAs) which consists of 10 amino acids and cannot be synthesized by animals that must be obtained from the diets. EAAs play an importance role in weight gain and growth performance in the consumers (De Silva and Anderson, 1995). All of three Thai fairy shrimps contain all 10 EAAs more than 60% of total amino acids, which are greater than the amounts of EAAs that were found in *Artemia salina* (31.1%) (Velu and Munuswamy, 2003).

Lipids are an important energy source for the development of animals (De Silva and Anderson, 1995). These three Thai fairy shrimps have lipid contents ranging from 6-10% similar to those of other commercial live foods, such as the brine shrimp *Artemia* sp. (9-13%) (Anh *et al*., 2009) and the cladoceran *Moina micrura* Kurz, 1874 (10.23%) (Das *et al*., 2007). Fatty acid is the most essential type of lipids that are effectively utilized in morphological changes in the larval development (De Silva and Anderson, 1995). Predominant fatty acids are found in all three species of Thai fairy shrimp; palmitic acid, oleic acid, stearic acid, linoleic acid and linolenic acid (Dararat *et al*., 2012). According to various studies that have indicated that fairy shrimps are rich in Polyunsaturated fatty acids (PUFAs), which are essential fatty acids for enhancement of growth and metabolism of various animals species (Mura *et al*., 1999; Arulvasu and Munuswamy, 2009). Recently, Thai fairy shrimps have been considered as a new type of live food in aquaculture (Sriputhorn and Sanoamuang, 2011). Beside, many authors have reported that fairy shrimps contain high levels of carotenoids (Velu and Munuswamy, 2007), which play an importance role in organism as biological antioxidants, protecting photo and lipid oxidation, as a source of vitamin A, enhance body coloration, increase reproductive performance, immunity and larval survival for aquatic animals (Nelis *et al*., 1988; Castillo and Negre-Sadargues, 1995; Linan-Cabello *et al*., 2002). Dararat *et al*. (2012) illustrated that all of three species of Thai fairy shrimps have quite high levels of carotenoid contents; *B. thailandensis* has the highest carotenoid contents (254.41 µg g^{-1} dry weight), followed by *S. siamensis* (211.92 µg g^{-1} dry weight) and *S. sirindhornae* (128.93 µg g^{-1} dry weight).

Hence, the presence of essential growth factors in alternative live food or Thai fairy shrimps were reported, efforts have been focused on the cultivation. Therefore, the study on life history and biological characteristics including growth, maximum size, life span, fecundity and hatchability will be conducted more understand and useful for the cultivation system of Thai fairy shrimp species. Dararat *et al*. (2011) revealed that among the three species, *B. thailandensis* has the largest size, shows rapid growth, and has the highest hatching percentage, which is suitable for mass production, while *S. sirindhornae* has the highest fecundity, which is appropriate for commercial production of eggs.

7. Stress proteins

The anostracans are fascinating animals that inhabit in unpredictable environments and experience stressful conditions. Hence, many anostracans have evolved interesting mechanisms, one of which is the production of resistant-shelled, dormant embryo, which is known as the cryptobiotic cysts. The resting eggs have several specialized biochemical adaptations by synthesis and accumulate biomolecules like glycerol, stress proteins, and alcohol-soluble carbohydrates (trehalose sugar) take place during physiological stress, which protect the protein from denaturation under extreme environmental conditions (Munuswamy *et al*., 2009).

Stress proteins are well known as thermotolerance proteins or heat shock proteins (HSP), which are a class of functionally related proteins involved in the folding and unfolding of other proteins. Their expression is increased when cells are exposed to extreme temperatures or other stresses, such as infection, inflammation, exercise, exposure to toxins, starvation, hypoxia (oxygen deprivation), nitrogen deficiency (in plants), or water deprivation. The dramatic up-regulation of the heat shock proteins is a key part of the heat shock response and is induced primarily by heat shock factor (HSF). Generally, HSPs are found in virtually all living organisms, from bacteria to human. Heat-shock proteins are named according to their molecular weight. For example, Hsp60, Hsp70 and Hsp90 (the most widely-studied HSPs) refer to families of heat shock proteins on the order of 60, 70, and 90 kDa in molecular weight, respectively. As a consequence, heat shock proteins function as intracellular chaperones for other proteins. They play an important role in protein interactions, which involve folding and assisting in the establishment of proper protein conformation (shape), prevention of unwanted protein aggregation, aid in transporting proteins across membranes within the cell and also stabilize partially unfolded proteins (Clegg, 2007).

Previous studies have been investigated that the occurrence of stress proteins in the primitive crustaceans using brine shrimp *Artemia* sp. as organism model because they are usually found in a wide variety of very harsh, hypersaline environments, the more than 500 described locations ranging from sea level to 4,500 m with fluctuating

temperatures (5-40 ^{o}C) and salinities (75-240 ppt) (Triantaphyllidis *et al*., 1998; Van Stappen *et al*., 1998). Thus, the active life history stages of this organism exhibit extraordinary osmoregulatory abilities and impressive resistance to thermal fluctuations. The most researches have been focused on *Artemia franciscana* from the San Francisco Bay which undergoes two alternative developmental pathways in life history. One is the path of direct development results in the release of fully-formed swimming nauplii from female and the alternative is the production of encysted gastrula embryos that enter to diapause termination and then released into the hypersaline environment (Clegg *et al*., 1999). In case of the diapauses destined or dormant embryos, which are confined by complex tolerant shelled. They are interrupted of development and extremely reduced metabolism (Drinkwater and Crowe, 1987; Drinkwater and Clegg, 1991; Clegg, 1997). Cysts can survive in extreme physiological stress including desiccation and prolonged anoxia (Clegg *et al*., 1999; Clegg, 2007). Whenever favorable conditions are presented, such as sufficient water, suitable temperatures and adequate levels of molecular oxygen, the encysted embryos can be resumed development (Drinkwater and Clegg, 1991). Interestingly, post-diapause development is characterized by the absence in both of DNA synthesis and cell division, until the embryo emerges from the cyst or hatches (Nakanishi *et al*., 1962, 1963; Olson and Clegg, 1978; Clegg and Conte, 1980). This unusual morphogenesis can also be interpreted as a significant adaptation because the embryos retain their resistant capabilities during post-diapause development, losing them only after the embryos emerge from their surrounding shells (Warner *et al*., 1997). The importance remarkably of this point is also significant that the level of stress protein p26 remains at a high level during post- diapause development, not being reduced to low levels until emergence occurs (Clegg *et al*., 1994). Generally, after the first stage nauplius molts, only a trace of p26 remains, and this is restricted to a few cells that do not decrease and eventually die (Liang and MacRae, 1999). Clegg *et al*. (1999) pointed out the correlation between the appearance of p26 in the diapause-destined embryo, and the cessation of DNA synthesis and cell division during development, allows for the interesting possibility that p26 might be involved in the inhibition of DNA synthesis and cell division, either as part of the protection of DNA and other nuclear constituents.

Massive amounts of two small stress proteins, p26 (Clegg *et al*., 1994) and artemin (De Herdt *et al*., 1979; De Graaf *et al*., 1990) are presented in stress-resistant encysted embryos using *Artemia* as organism model. Each protein makes up 10-15% of the total non-yolk proteins 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). Clegg *et al*. (1999) examined p26 and artemin in a wide variety of invertebrates including the resting stages of other closely related crustaceans. But, there are neither p26 and artemin were detected by western blotting in cysts of fairy shrimp, *Branchinecta sandiegonesis* a closely related species of *Artemia* (Spears and Abele, 2000), cysts of the notostracans *Triops* sp. or ephippia of the cladoceran, *Daphnia* sp. Furthermore, Coomassie staining failed to reveal significant amounts of protein anywhere near 26 kDa in above samples. Whereas, abundantly documented in several strains of *Artemia*, like *Artemia franciscana* (Sanfrancisco Bay area), *Artemia sinica* (China), *Artemia parthenogenetica* (Siberea and France), *Artemia urmiana* (Iran), *Artemia salina* (Tunisia) and *Artemia monica* (USA). At this point, there is no evidence for the presence of p26 and p26-like protein or artemin outside the genus of *Artemia* (Clegg *et al*., 1999). Munuswamy *et al*. (2009) investigated the interesting result that p26 and artemin-like protein were detected in cysts of freshwater fairy shrimp *Streptocephalus dichotomus*, unlike former freshwater fairy shrimps. Thus, it seems possible that *S. dichotomus* cysts are able to repair much of the damage to macromolecules upon rehydration, same as *Artemia*. Earlier studies on *Artemia* have been reported that p26 is produced in oviparous developing embryos and is not found in ovoviviparous developing embryos, but no difference was found between the desiccated and non-desiccated cysts of *S. dichotomus*. Hence, this is the spectacular occurrence of p26 and artemin-like protein in freshwater from other than *Artemia*. So, the life cycle of *S. dichotomus* has been outlined for representing the occurrence of p26 and artemin in both of during the production of diapause embryos and spontaneous development, which fertilized eggs directly hatch into nauplii. It is also significant that the level of p26 remains high, not being reduced to low levels until emergence occurs, according to the synthesis of artemin, which is up-regulated strongly during aerobic recovery after extended anoxia (Clegg *et al*., 1999). The protein profiles of stress proteins in *S. dichotomus* were detected using western blot and Coomassie stain technique. Both of the spontaneous (non-desiccated) and desiccated cysts gave

similar results when observed the occurrence of protein in the homogenate, supernatant and pellet fraction. Interestingly, p26 proteins were found in the homogenate, supernatant and pellet fraction, while artemin-like proteins were strongly found in the homogenate and the fractions of supernatant, but not in the pellet (Munuswamy *et al*., 2009). As the result, illustrated that p26 and artemin play an important role for the resistant stress conditions of encysted embryos, but they are slightly difference key function in embryonic cells. Clegg *et al*. (1999) discussed in the features of p26, which is a small heat shock protein/ α-crystallin protein that the native protein has molecular mass of about 700 kDa (27 sub-units, each about 26 kDa) and appears as a particle of about 15 nm in a diameter under the electron microscope. Stress proteins p26 also present only in the encysted embryo where it accumulates to a massive 10-15% of the total non-yolk proteins. The synthesis of p26 has not been induced by stress under any of various conditions examined, but instead is programmed only in embryos destined to enter diapause, thus the accumulation of this protein indicated that the embryos may experience for years or even decade later. When activated encysted embryos are exposed to anoxia or thermal shock, about half of the p26 in the cytoplasm is translocated into the nucleus, and is reversed when the stress is removed. This evidence suggests that p26 also interacts with a wide variety of cytoplasmic proteins in anoxic embryos, and these interactions are reversed when aerobic conditions are restored. Stress protein p26 is called as nuclear/cytoplasmic translocation protein, which shows strong pH dependence *in vitro*: acidic pH favors translocation into nuclei, and that is reversed by alkaline pH. One of several interesting feature is the absence of a typical nuclear localization signal, in spite of its extensive translocations between cytoplasm and nucleus. Furthermore, recent studies have been investigated the characteristics of p26, which carried out more clearly understand in both of structure and function of stress protein p26. Small heat shock/ α-crystallin protein or p26 are defined by conserved sequence of approximately 90 amino acid residues, which termed as α-crystallin domain. Sequence modeling and physical analyses show the secondary structure of small heat shock/α-crystallin proteins that is predominately beta-pleated sheet. The flexible carboxy-terminal extension contributes to chaperone activity by enhancing the solubility of small heat shock/α-crystallin proteins (MacRae, 2000). In the context, Clegg *et al*. (1999) found that the small heat shock/α-crystallin protein p26 undergoes nuclear translocation in

response to stress in encysted embryos of the brine shrimp *A. franciscana*. About 50% of total p26 translocate to nuclei in embryos treated with heat shock or anoxia, and in embryo homogenates incubated at low pH. Nuclear fractionation shows that the majority of nuclear p26 and a nuclear lamin are associated with the nuclear matrix fraction. To further explore the roles of p26 and other heat shock proteins (HSPs) in stabilizing nuclear matrix proteins (NMPs), Willsie and Clegg (2002) examined the nuclear matrices from control, and heat-shocked embryos, which were disassembled in urea and evaluated by two-dimensional (2-D) gel electrophoresis and western immunoblot analysis. The results show that nuclear lamins were present only in assembled fractions (control), while p26 and HSP70 were also present in the heat shocked embryos. Moreover, confocal microscopy on isolated nuclei and nuclear matrix preparations from control and heat-shocked embryos show that the majority of p26 and nuclear lamins share similar nuclear distributions. The combination of microscopy and fractionation results suggested that p26 and HSP70 play a role in the protection of nuclear lamins within the nuclear matrix (Willsie and Clegg, 2002). In addition, Derham and Harding (1999) examined the translocation of p26 into nuclei of heat shock or anoxia conditions *in vivo* and acid pH *in vitro*. The results suggested that p26 are responsible for the reduced transcription rates involving intracellular pH (pH_i) decrease, which is the reason for down-regulated RNA synthesis and protein synthesis in intact embryos recovering from heat shock or anoxia conditions. Confocal microscopy confirmed that p26 moves into nuclei in response to heat shock and anoxia *in vivo*, and to low pH *in vitro*, and indicated that the nuclear distribution of p26 is similar under all three conditions. Hence, this evidence determined that unstressed embryos containing p26 in all their nuclei will not hatch, even under permissive conditions (Derham and Harding, 1999).

Previous studies point out the another protein that is abundant as p26, which is called as artemin. It is mostly co-occurring with p26 in the encysted embryos. Artemin exhibits substantial resemblance in amino acid sequences to the ferritins, but does not contain iron. Artemin makes up10-15% of the total non-yolk protein of resting cyst and has not been detected in other life cycle stages as same as p26 (Chen *et al.*, 2003). Because p26 plays an important role as a molecular chaperone of proteins in stress-

resistant embryos (Clegg *et al*., 1999), it seemed possible that artemin contributes similarly, so Warner *et al*. (2004) undertook such a study and investigated this context that apo-artemin is thermostable protein with RNA-binding ability, and found only in stress-resistant embryos destined for deep dormancy (diapause), suggesting that this protein might function as an RNA chaperone *in vivo* according to Clegg *et al.* (1999) have been suggested that a plausible function for artemin is that it stabilizes or otherwise protects mRNA during the long-term dormancy and severe stress, which these embryos normally experience in nature, including high temperature. Thus, both of artemin and the well-described molecular chaperone, p26, seem to be important components of the adaptive repertoire of the encysted embryos that ensures their survival under the stress environmental conditions that they face in nature.

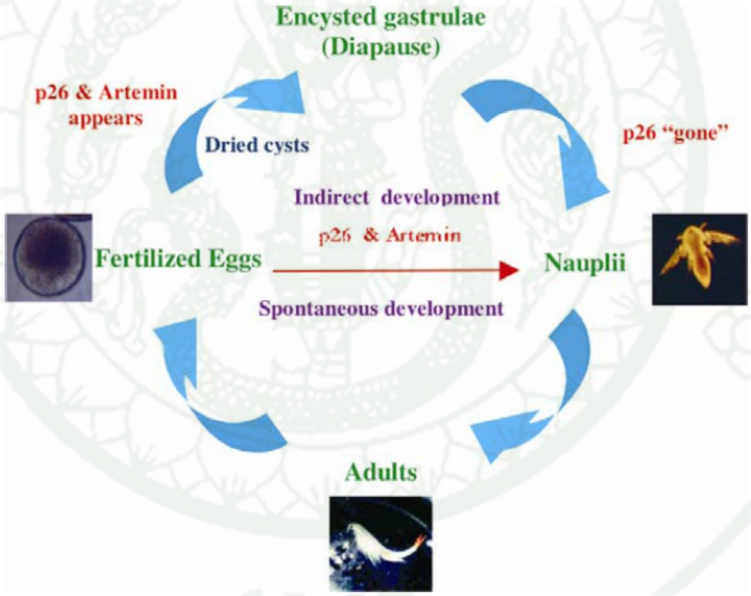

Figure 2 Schematic diagram showing the occurrence of p26 and artemin-like proteins in the life cycle of *Streptocephalus dichotomus*.

Source: Munuswamy *et al*. (2009)

8. Decapsulation procedure

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). Nowadays, brine shrimp (*Artemia* sp.) and fairy shrimp are commonly used in feeding larval or young aquatic animals. Indeed, cysts or eggs of most brine shrimps and fairy shrimps have the thick shell, which cannot be digested by predators and may cause blockage of the gut and other deleterious effects (Sorgeloos *et al*., 1977). Additionally, the shells tend to cause water pollution and act as a carrier of disease, which carry spores of bacteria, plant, and even small animal species (Tunsutapanich, 1979). Thus, the decapsulation procedure was conducted to solve above problems and applied for further studies, which can be completely removed hard shell for the short-time. Sorgeloos *et al*. (1977) offered the advantages of decapsulation method 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 and 3) possible direct ingestion and digestion to decapsulated cyst by fish and crustacean larvae, which has a smaller particle size and reduced the deleterious effects. There are two common methods (chlorine and chlorine/sodium hydroxide method) used to decapsulate the cysts. The first step of both methods is to hydrate the cysts by soaking them in either fresh or salt water for about an hour, then decapsulate using chemical substances (chlorine and chlorine/sodium hydroxide). For chlorine method, used household bleach (6% sodium hypochlorite solution) or industrial chlorine (11% sodium hypochlorite solution) for approximately 3- 5 minutes or until the color of cysts will change from dark brown to gradually white or orange. Then, thoroughly wash with fresh or sea water in order to remove all trace chemicals and without smell of chlorine remains. The alternative is chlorine/sodium hydroxide method, which is used 50% sodium hydroxide (50% NaOH) and industrial chlorine (11% sodium hypochlorite solution) and should be performed in low temperature (4-7 ^{o}C) to prevent heat damage to the cysts for about 3-5 minutes and washed thoroughly until chemicals clearance (Aquatic Eco-systems, 1979). Furthermore, Tunsutapanich (1979) offered the alternative method, which introduced new chemical substances for using in the decapsulation process. The result of this experiment proved that chemical substances are cheaper and more readily available

in Thai market, such as lime (CaO) and bleaching powder (calcium hypochlorite). In addition, the decapsulation procedure will be conducted to investigate clearly embryonic developmental features and have better hatchability than non-decapsulated cysts.

MATERIALS AND METHODS

1. Sources of cysts

Cysts of *Branchinella thailandensis* were obtained from Faculty of Agriculture and Natural Resources, Rajamangala University of Technology Tawan-ok (RMUTTO), Bangpra campus, Chonburi province. Cysts of *Streptocehalus sirindhornae* were obtained from Faculty of Fisheries, Suphanburi College of Agriculture and Technology (SPCAT), Suphanburi province. They were kept in plastic zip sealed bags and stored in refrigerator.

2. Scanning electron microscopy

Whole cysts and cross-sectioned cysts using a fine surgery knife were taken to study surface topography and internal topography of cysts, respectively. Cysts were incubated in the laboratory conditions by exposure to the continuous illumination of approximately 4,500 Lux at room temperature (25-27 ^{o}C) for 12 hours. Nauplii have hatched and were fixed in 2.5% glutaraldehyde prepared in sodium cacodylate buffer (pH 7.4) at 4^{o}C. The fixed samples were washed in distilled water and dehydrated through series of acetone as followed 20%, 40%, 60%, 80% and three times of 100% acetone concentration, then critical point-dried using CO_2. Three types of specimens (whole cysts, cross-sectioned cysts and nauplii) were mounted on standard metal stubs, coated with gold (< 10 nm) and observed with a JEOL JSM-5600LV scanning electron microscope at a voltage of 10 kV, placed at Kasetsart Agricultural and Agro-Industrial Product Improvement Institute.

3. Decapsulation and hatching efficiency assay

The cysts of *B. thailandensis* and *S. sirindhornae* were hydrated in de-chlorinated water for 30 minutes. Hydrated cysts were decapsulated using sodium hypochlorite, which contained 1%, 2%, 4% and 6% of active ingredients of sodium hypochlorite (commercial brand “Haiter bleach” contained 6% of active ingredients)

and stirred for five minutes until completed decapsulation reaction, which 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 decapsulated cysts of four treatments (1%, 2%, 4% and 6% of sodium hypochlorite) and non-decapsulated cysts or whole cysts (controlled group) were incubated for five replications 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 ^{o}C) for hatching. Hatched eggs were counted at 6, 8 and 24 hours under stereomicroscope. Data were transformed to the percentage hatchability and analyzed by analysis of variance (One-way ANOVA). Significant differences (p<0.05) were discovered by Duncan's multiple range tests.

4. Embryonic development at different timescales

The cysts of *B. thailandensis* and *S. sirindhornae* were hydrated in de-chlorinated water for 30 minutes. Hydrated cysts were decapsulated using 2% of sodium hypochlorite (the optimal concentration) for five minutes and washed thoroughly with de-chlorinated water for removing chemicals. Decapsulated cysts were incubated in 10 ml of de-chlorinated water in 2x3 multi-well plates with continuous illumination of 4,500 Lux at room temperature (25-27 ^{o}C) for hatching. Samples were randomly selected every 30 minutes until to 8 hours and fixed in 95% ethanol. Fixed samples were immersed in de-chlorinated water for 1 minute and dropped on glass slides with two drops of 100% glycerine. Slides were mounted and then observed using light microscope. The developmental embryos of each time (30 minutes to 8 hours) were taken photograph at 40x of power magnification and described the primary characteristics, which compared by the criteria of Munuswamy *et al*. (2009).

5. Cell-free extracts

The desiccated cysts of *B.thailandensis* and *S. sirindhornae* were homogenized at 60 mg dry weight embryos/ml of 1xPBS buffer using plastic homogenizers. The homogenates were prepared for two fractions (approximately 3 ml per fraction). A fraction of homogenates was directly used and the other one was centrifuged at 25 ºC, 1630 g for five minutes, supernatants were removed from resulting pellets. The pellets were washed with 1xPBS buffer and re-centrifuged at 25 ºC, 1630 g for five minutes and restoring the original volume using 1xPBS buffer. Aliquots of homogenates (h) supernatants (s) and pellets (p) fractions were added to 1 part of 4x sample buffer (Laemmli sample buffer) with 3 parts of samples and heated at 100 ºC for five minutes. Supernatants and pellets fractions were centrifuged at 25 ºC, 600 g for five minutes.

6. SDS-PAGE procedure

The split (10 µl) of extracted fractions (homogenates, supernatants and pellets) and biotinylated markers were loaded onto 12% SDS-polyacrylamide gel (12% Mini-PROTEAN TGX™ Precast Gel) for electrophoresis at 160V and 60 mA using Bio-Rad's mini-Protean II system for 1 hour. Protein bands on gel were detected with Coomassie Brilliant Blue R-250 for 30 minutes and destained with 25% methanol in 7% glacial acetic acid for 2-3 times. Destained bands were exposed to the Gel-Doc.

RESULTS AND DISCUSSION

Results

1. Scanning electron microscopy

The scanning electron micrographs revealed the attributes of cysts of both Thai fairy shrimps *B. thailandensis* and *S. sirindhornae*. Cyst of *B. thailandensis* shows spherical shape with a regular pattern of pentagons and hexagons including appearance of ridges with sloping sides and depression regions (Figure 3A). Diameter of cyst is approximately 200 μm. The external ornamentation is made up of pores and spiny projections (Figure 3B,C). The structural morphology of cross-sectioned demonstrated three layers of egg shell (Figure 3D). The innermost layer enveloping the embryo is the cuticular layer or embryonic cuticle (~2-3 μm), which remained in the decapsulated cysts under light microscope. The middle layer is the alveolar layer (~5-7 μm) with small variable size hollows and the outermost of egg shell is defined as the thick outer cortex (~25-30 μm).

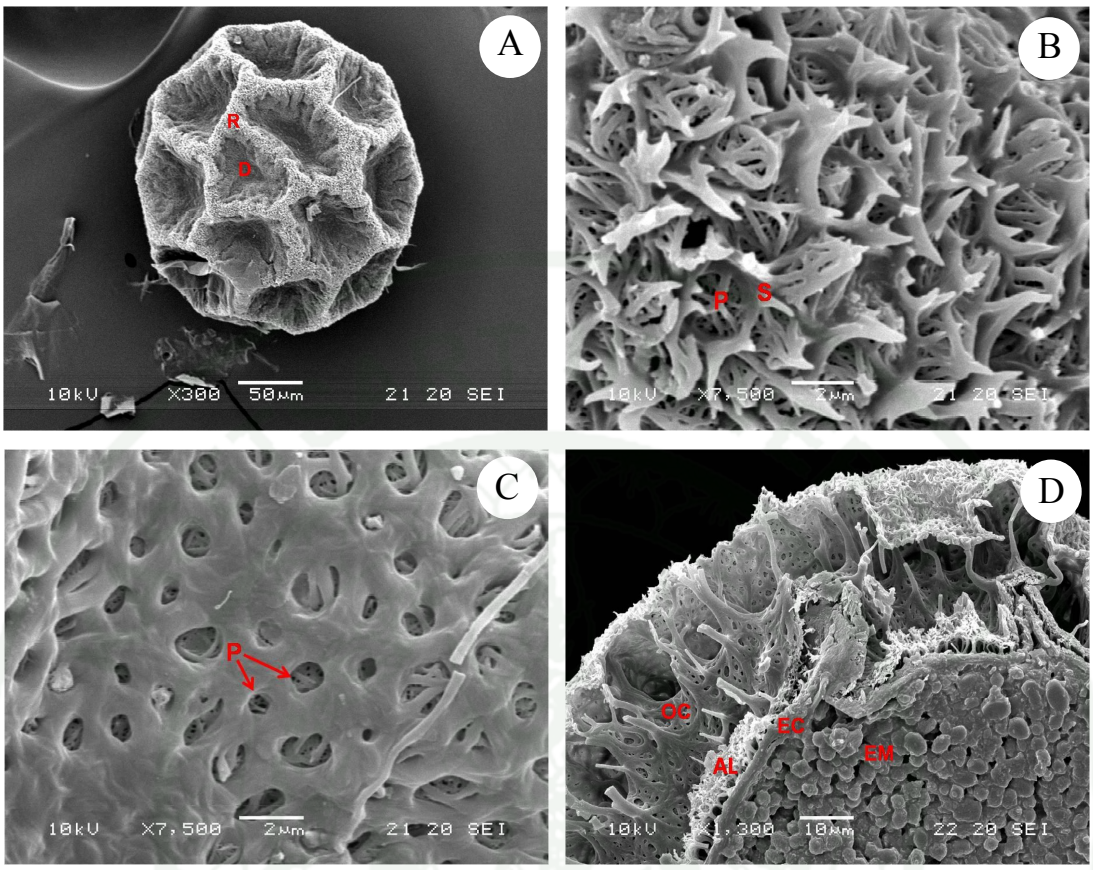

Figure 3 Scanning electron micrographs of *Branchinella thailandensis*. **A**, Cyst of *B. thailandensis* shows spherical shape with a regular pattern of polygons. **B**, Surface topography of ridge shows pores and spiny projections. **C**, Pores on surface of the depression region. **D**, Cross-sectioned of desiccated cyst. R; ridge, D; depression, P; pore, S; spine, EM; embryonic mass and yolk granules, EC; embryonic cuticle, AL; alveolar layer, and OC; outer cortex.

Cyst of *S. sirindhornae* shows spherical shape with a regular pattern of variable polygons including pentagons and hexagons. There is also appearance the ridges with sloping sides and depression regions (Figure 4A). Diameter of cyst was smaller than cyst of *B. thailandensis* which is approximately 150-180 µm. Pores on sculptured surface of egg shell are prominent but disappear the spiny projections (Figure 4B,C). Cross-sectioned of cyst shell composes of indistinct three layers (Figure 4D).

The innermost layer enveloping the embryo is the cuticular layer or embryonic cuticle (~2 µm). The middle layer is the alveolar layer (~10-15 µm) which is characterized by an alveolar mesh and large lacunae, and also not demarcated with inner layer which appeared small variable size lacunae and the outermost is the outer cortex (~5 µm).

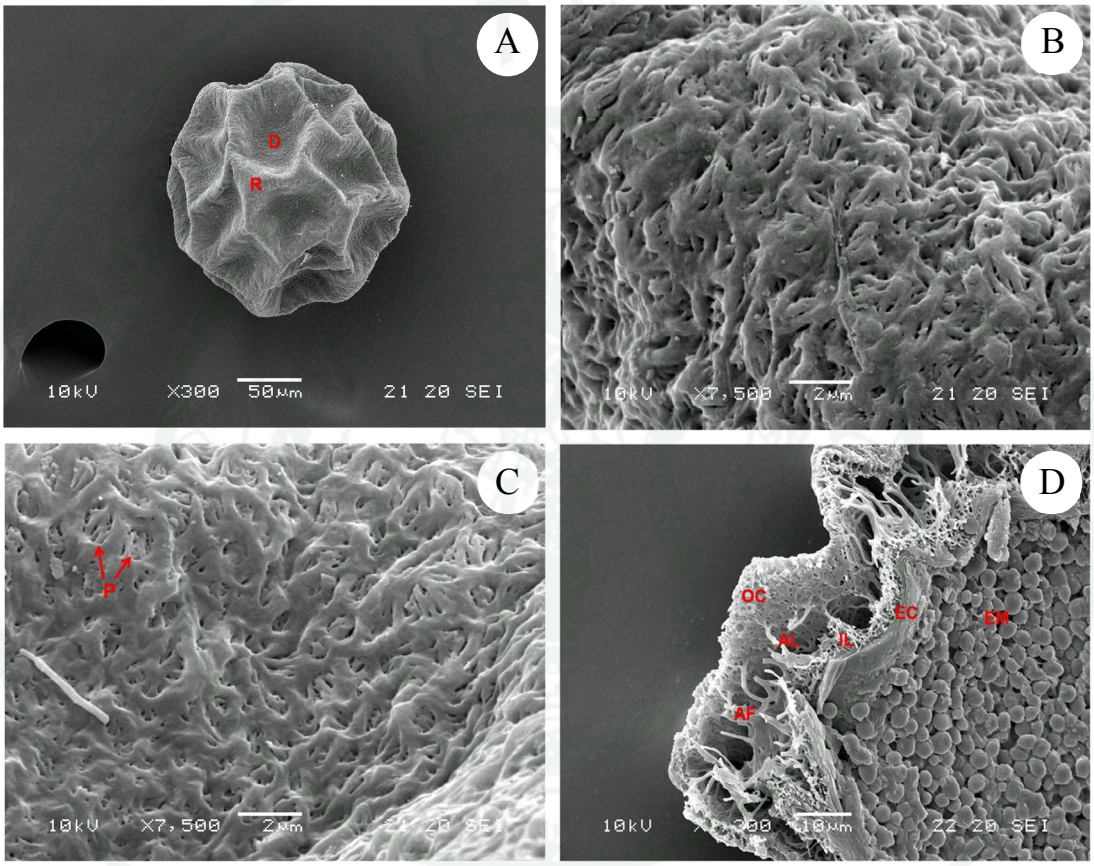

Figure 4 Scanning electron micrographs of *Streptocephalus sirindhornae*. **A**, Cyst of *S. sirindhornae* shows spherical shape with a regular pattern of polygons. **B**, Surface topography of ridge shows pores. **C**, Pores on surface of the sloping side and depression region. **D**, Cross-sectioned of desiccated cyst. R; ridge, D; depression, EM; embryonic mass and yolk granules, EC; embryonic cuticle, AF; alveolar fiber, AL; alveolar layer, IL; inner layer and OC; outer cortex.

The scanning electron micrographs of nauplii of the two species fairy shrimps revealed the characters on the larval morphology which incubated for 12 hours. Nauplii of *B. thailandensis* exist approximately 400-450 μm in length. The three naupliar appendages, antennules, antennae and mandibles are well developed and functional as well as anal pore. Dorsal view of nauplius represents dorsal organ which covers the functional cephalic region corresponding to the antennae and mandibles (Figure 5A). Ventral view shows the labrum and the details of appendages processes (Figure 5B). The labrum is large, inflated and appeared U-shaped. It overhangs at the median tips of the mandibular coxae and lacks of setae. The first antennae or antennules are inserted anteriorly and also laterally which separated by a space of the width of the labrum. They are long, slender, unsegmented and bear three distal setae. The second antennae are located on the both lateral sides of labrum. They are large which almost appear the half length as the whole animal and biramous. The protopod is gradually divided into a coxal and basipodal part. The coxal portion has a large spine oriented towards the mouth. The basipod also bears another spine directed to the midline of the animal. The distal portion of antenna is divided into two sections endopod and exopod. The endopod is smaller than the exopod, also unsegmented and bears three setae directed to the midline of the body. The exopod has about the length of the protopod and hold the arranged setae. The mandibles are inserted laterally on the body. It consists of an enlarged coxa with the three segmented palps. The coxa is weakly observed which is covered by the fleshly U-shaped labrum. The palp also bears setae processes which directed to the midline ventral body. The thoracic-abdominal part is seen as a cone shaped. The trunk is weakly demarcated and almost occurred 4-5 segments of trunk lime buds. Actually, there is a pair of short and segmented-like sections, corresponding to the maxillules and maxillae which locate behind of the naupliar head portion, but they are hidden by the long U-shaped labrum. The anal pore is completed opening and also well developed caudal rami in the distal posterior region.

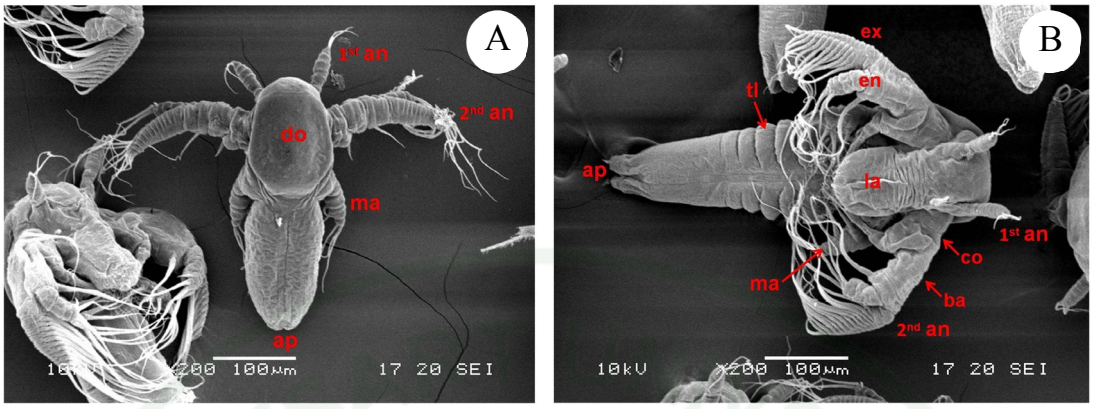

Figure 5 Nauplius of *Branchinella thailandensis*. **A**, Dorsal view. **B**, Ventral view. 1st an; the first antennae or antennules, 2nd an; the second antennae, ma; mandibles, do; dorsal organ, ap; anal pore, la; labrum, co; coxa, ba; basal, en; endopod, ex; exopod and tl; trunk lime.

The early naupliar larva of *S. sirindhornae* is approximately 250-300 µm in length. Nauplius exists three developed appendages, antennules, antennae and mandibles. Dorsal view of nauplius represents round dorsal organ which covers functional cephalic regions (Figure 6A), whereas ventral view shows the labrum and the details of appendages processes (Figure 6B). The labrum is large, inflated and appeared U-shaped. It locates at the median tips of the mandibular coxae and lacks of setae. The first antennae or antennules are inserted anteriorly and also laterally which separated by the labrum. They are long, slender, unsegmented and bear three setae at the tips. The second antennae are located on the both lateral sides of labrum. They are large which almost appear the same length as the whole animal and biramous. The protopod is slightly divided into a coxal and basal part. The coxal portion has a large shortest spine. The basipod also bears another spine directed to the body. The distal portion of antenna is divided into endopod and exopod. The endopod is smaller than the exopod, also unsegmented and bears three setae directed to the midline of the body. The exopod has about the length of the protopod and hold the arranged setae. The mandibles are inserted laterally on the body. It consists of an enlarged coxa with the three segmented palps. The palp also bears setae processes which directed to the midline ventral body. The trunk is still not demarcated. The thoracic-abdominal

region is observed egg like shaped. The anal pore is completed opening while still not developed caudal rami in the posterior region.

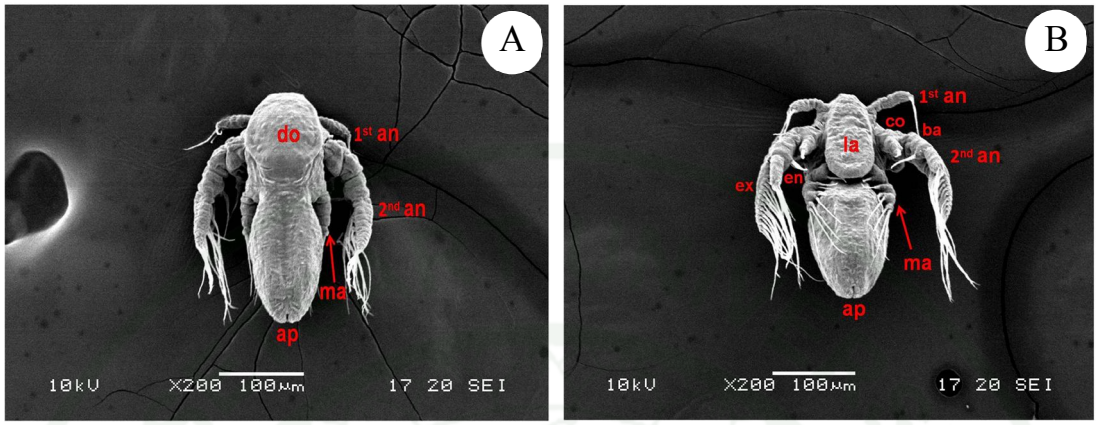

Figure 6 Nauplius of *Streptocephalus sirindhornae*. **A**, Dorsal view. **B**, Ventral view. 1st an; the first antennae or antennules, 2nd an; the second antennae, ma; mandibles, do; dorsal organ, ap; anal pore, la; labrum, co; coxa, ba; basal, en; endopod and ex; exopod.

2. Decapsulation and hatching efficiency

The hatching efficiency data of the two Thai fairy shrimps *Branchinella thailandensis* and *Streptocephalus sirindhornae* are represented in the relationship between percentage hatchability and continuing three observed times (6, 8 and 24 hours). The results are also shown in the comparison of percentage hatchability between whole cysts or non-decapsulated cysts which denoted as controlled group and decapsulated cysts using four treatments of sodium hypochlorite concentration levels (1%, 2%, 4% and 6%). The hatching percentages of *B. thailandensis* and *S. sirindhornae* are calculated and summarized in the Figure 7 and 8, respectively.

Attentive observation on egg hatching of *B. thailandensis* for continuous incubation times (6, 8 and 24 hours) investigated that eggs of controlled group hatched as the highest rate which represented 20.84%, 31.60% and 50.70%, while decapsulated cysts using 6% of sodium hypochlorite showed the lowest hatching rate, as followed 0.23%, 0.43% and 1.04% through 6, 8 and 24 hours, respectively.

The hatching efficiency of all treatments increased significantly ($p<0.05$) when increasing incubation times. After 6 hours of hydration, the hatching rate was not significantly different ($p>0.05$) between whole cysts (20.84%) and decapsulated cysts using 1% of sodium hypochlorite concentration (20.29%) including 2% of sodium hypochlorite decapsulated cyst treatment (20.44%), whereas strong significant difference ($p<0.05$) with 4% of decapsulated cyst treatment (8.40%) and 6% of decapsulated cyst treatment (0.23%). In addition, there were significant difference ($p<0.05$) in the percentage hatchability of 4% decapsulated cyst treatment and 6% decapsulated cyst treatment. After 8 hours of incubation, the percentage hatchability was not significantly different ($p>0.05$) between controlled group (31.60%) and decapsulated cysts using 1% of sodium hypochlorite (31.25%) also with 2% of sodium hypochlorite decapsulated cyst treatment (31.14%) but significantly differ from 4% of sodium hypochlorite (14.75%) and 6% of sodium hypochlorite decapsulated cyst treatment (0.43%). There were significant differences ($p<0.05$) in hatching efficiency among 4% of sodium hypochlorite and 6% of sodium hypochlorite decapsulated cyst treatment. After 24 hours of incubation, there were no significance differences ($p>0.05$) between controlled group (50.70%) and 1% of sodium hypochlorite (48.44%), also including 2% of sodium hypochlorite decapsulated cyst treatment (48.88%). Furthermore, the hatching rate data showed the significant difference ($p>0.05$) between controlled group and 4% of sodium hypochlorite (20.97%) and 6% of sodium hypochlorite decapsulated cysts (1.04%). There were also significant differences ($p<0.05$) in hatching rate among 4% of sodium hypochlorite and 6% of sodium hypochlorite decapsulated cyst treatment.

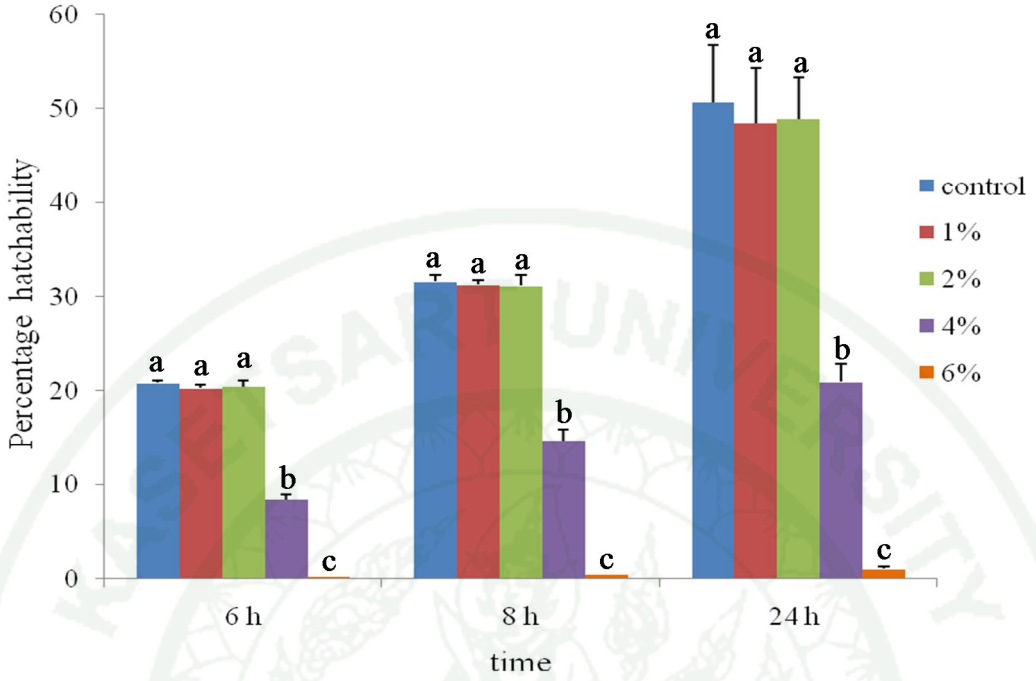

Figure 7 Percentage hatchability of *Branchinella thailandensis* (Mean±SD. with the same letter are not significantly different from each other, Duncan's test, $p<0.05$)

The hatching efficiency data of *S. sirindhornae* showed the highest hatching rate in eggs of controlled group which appeared 7.35%, 12.51% and 21.95%, whereas decapsulated cysts using 6% of sodium hypochlorite concentration displayed the lowest hatching rate as followed 0.19%, 1.37% and 2.80% through 6, 8 and 24 hours, respectively. The percentage hatchability of all treatments presented the tendency significance increasing ($p<0.05$) when increasing incubation times. After 6 hours of hydration, the hatching rate was not significantly different ($p>0.05$) between controlled group (7.35%) and decapsulated cysts using 1% of sodium hypochlorite concentration (7.08%) including 2% of sodium hypochlorite decapsulated cyst treatment (7.07%), whereas gender significant difference ($p<0.05$) existed in 4% of decapsulated cyst treatment (3.13%) and 6% of decapsulated cyst treatment (0.19%). However, there was significant difference ($p<0.05$) in the percentage hatchability among 4% of decapsulated cyst treatment and 6% of decapsulated cyst treatment. After 8 hours of

incubation, the percentage hatchability was not significantly different (p>0.05) between controlled group (12.51%) and decapsulated cysts using 1% of sodium hypochlorite (12.18%) also with 2% of sodium hypochlorite decapsulated cyst treatment (12.09%), but significantly differ from 4% of sodium hypochlorite (7.32%) and 6% of sodium hypochlorite decapsulated cyst treatment (1.37%). There was gradually significant differences (p<0.05) in the hatching efficiency among 4% of sodium hypochlorite and 6% of sodium hypochlorite decapsulated cyst treatment. After 24 hours of incubation, there was no significance differences (p>0.05) between controlled group 21.95% and 1% of sodium hypochlorite (21.37%), also including 2% of sodium hypochlorite decapsulated cysts (20.90%). In addition, the hatching rate data showed the distinguishable significance (p<0.05) between controlled group and 4% of sodium hypochlorite (11.03%) and 6% of sodium hypochlorite decapsulated cysts (2.80%). There was also considerable differences (p<0.05) in the hatching rate among 4% of sodium hypochlorite and 6% of sodium hypochlorite decapsulated cyst treatment.

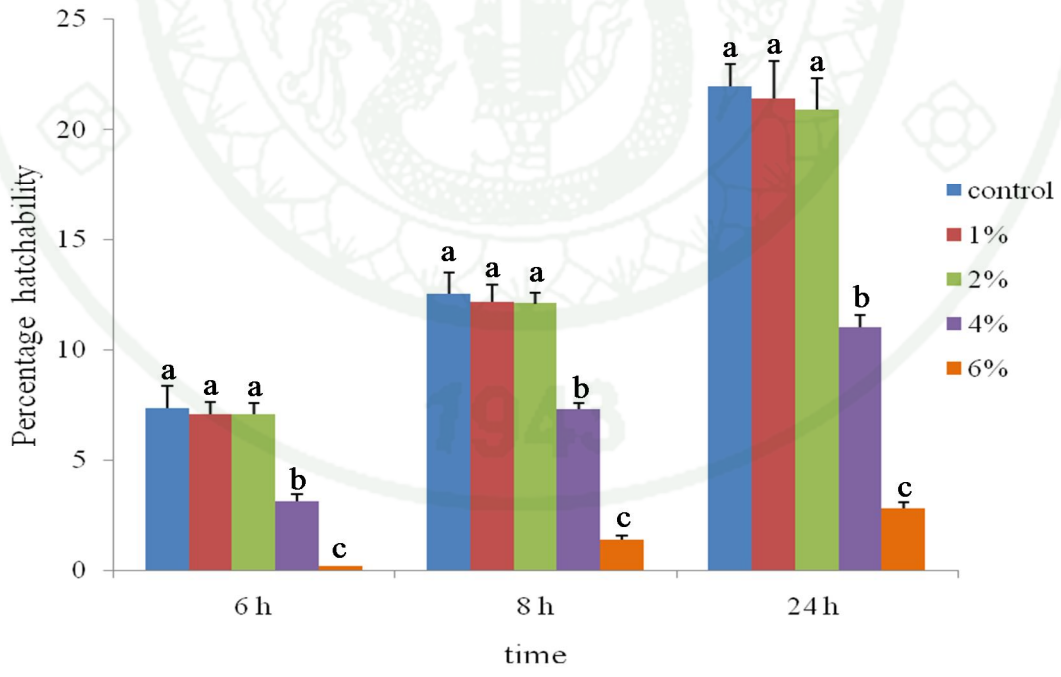

Figure 8 Percentage hatchability of *Streptocephalus sirindhornae* (Mean±SD. with the same letter are not significantly different from each other, Duncan's test, p<0.05)

As the following results, indicated that the decapsulated cysts using 1% and 2% of sodium hypochlorite concentration represented the sameness hatching efficiency with controlled group or whole cysts. Hence, both of 1% and 2% of sodium hypochlorite concentration can be used for releasing the external shell of eggs. However, the most clearance substance was 2% of sodium hypochlorite which clearly decapsuled the chorion of cysts without affecting the viability of embryos.

3. Embryonic development at difference timescales

Embryonic development of the two Thai fairy shrimps *B. thailandensis* and *S. sirindhornae* are observed ongoing continuous hydration since the first 30 minutes until 8 hours. The developmental process indicates the morphogenesis of developing embryo to fully formed nauplius, which is a larval form with three pairs of appendages and a single median naupliar eye.

Morphoogenesis and embryonic development of *B. thailandensis* takes place to the fully formed nauplius within 8 hours after hydration. The light microscopic observations revealed the thorough occurrence of developmental process through time changing. The decapsulated cyst after 30 minutes of hydration clearly shows embryonic mass and prominent yolk granules in the developing embryo (Figure 9A). After 1 hour of incubation, the embryo is still undifferentiated and the yolk granules are gradually utilized (Figure 9B). After 1 hour 30 minutes, yolk granules are gradually utilized for developing, subsequently the embryo within the embryonic cuticle will be divide into two hemi-halves (Figure 9C). After 2 hours, the embryo divides into two hemi-halves to differentiate into the anterior and the posterior regions, by that the anterior part is broader than the posterior part (Figure 9D). After 2 hours 30 minutes, signs of morphogenesis occur as showing the development of larval limb buds (Figure 9E). After 3 hours, the anterior region develops into the brown head and the posterior region as the thoracic and abdominal region. Furthermore, the appendages will be developing (Figure 9F). After 3 hours 30 minutes, the embryo within the embryonic cuticle is further developing and showing beside appendages (Figure 9G). After 4 hours, substantially changing occurred in the embryo which developing the antennae and

mandibles together with beginning to appear the median naupliar eye (Figure 9H). After 4 hours 30 minutes, obvious occurrence of the median naupliar eye and continuous developing of antennae and mandibles (Figure 9I). After 5 hours, appearance of development completed to the nauplius stage, which the first and the second antennae and mandibles develops as well as the anal pore, followed by hatching (Figure 9J). Since after 5 hours 30 minutes of hydration, which is defined as nauplius further develops and just hatches from the embryonic cuticle into free swimming nauplius (Figure 9K-P).

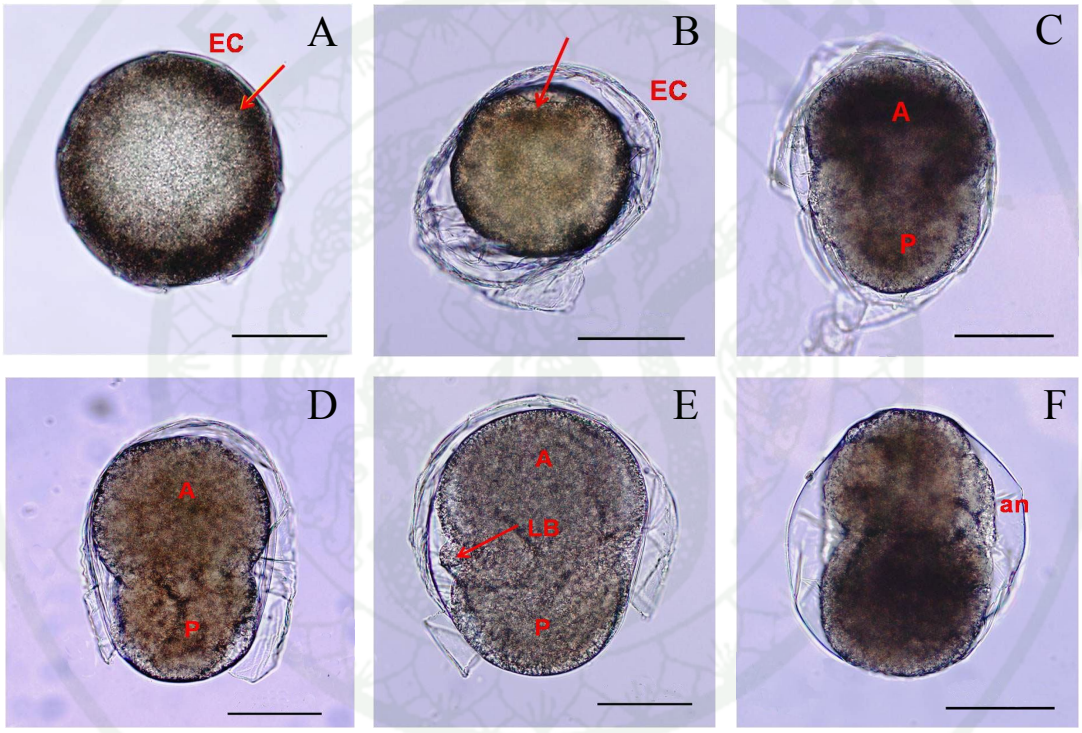

Figure 9 Embryonic development of *Branchinella thailandensis* (Scale bar = 200 µm). **A**, After 30 min, the cyst clearly shows prominent yolk granules. **B**, After 1 h, the yolk granules are gradually utilized (arrow). **C**, After 1 h 30 min, the embryo will be divide into two hemi-halves. **D**, After 2 h, two hemi-halves differentiate into the anterior and the posterior regions. **E**, After 2 h 30 min, embryo shows the development of larval limb buds. **F**, After 3 h, the anterior part develops into a head and the posterior as the thoracic-abdominal region. EC; embryonic cuticle, LB; limb bud, A; anterior part, P; posterior part, an; antennae.

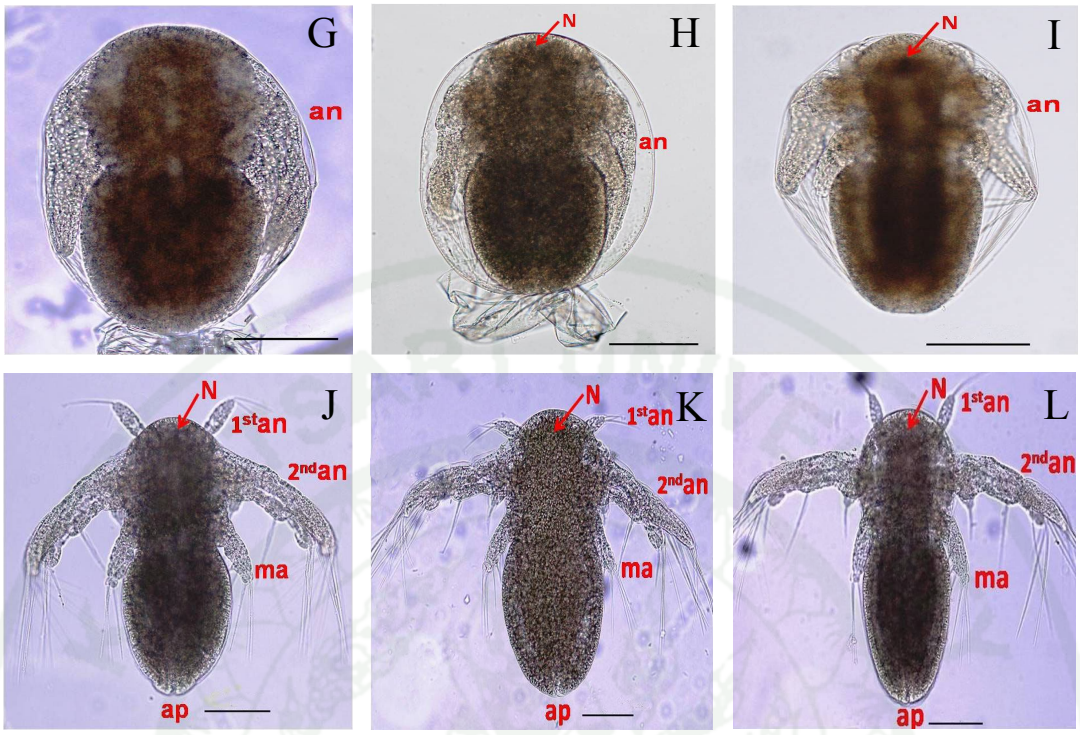

Figure 9 (Continued) **G**, After 3 h 30 min, the embryo is further developing and showing beside appendages. **H**, After 4 h, embryo develops the antennae and mandibles together with the median naupliar eye. **I**, After 4 h 30 min, continuous developing the appendages. **J**, After 5 h, completed development to the nauplius including well-developed the anal pore. **K**, After 5 h 30 min, the nauplius hatches from the embryonic cuticle into free swimming nauplius. **L**, After 6 h, the nauplius hatches from the embryonic cuticle into free swimming nauplius. an; antennae N; median naupliar eye, 1st an; the first antennae, 2nd an; the second antennae, ma; mandibles, and ap; anal pore.

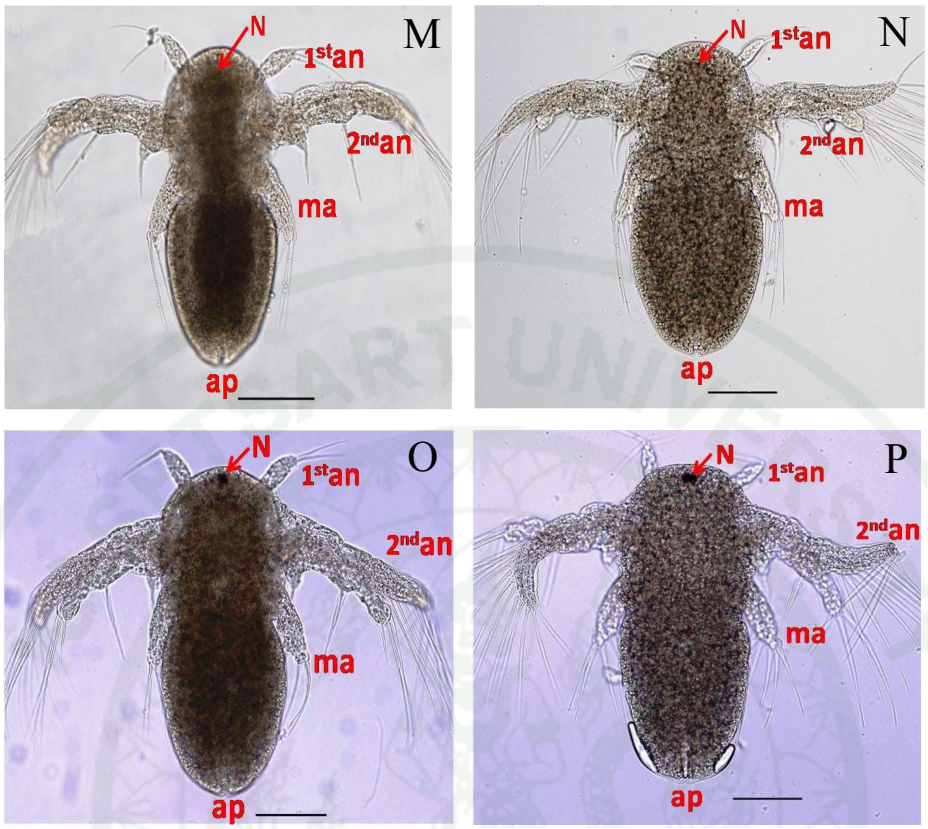

Figure 9 (Continued) **M-P**, After 6 h 30 min to 8 h, the nauplius hatches from the embryonic cuticle into free swimming nauplius. N; median naupliar eye, 1st an; the first antennae, 2nd an; the second antennae, ma; mandibles, and ap; anal pore.

After incubation for 30 minutes, decapsulated cyst of *S. sirindhornae* clearly shows prominent yolk granules as same as *B. thailandensis* (Figure 10A). After 1 hour, embryo is still undifferentiated and distinct appears yolk granules (Figure 10B). After 1hour 30 minutes, yolk granules are gradually utilized and the embryo will be divide into two hemi-halves (Figure 10C). After 2 hours, embryo divides into two hemi-halves (Figure 10D). After 2 hours 30 minutes, embryo clearly differentiate into the anterior and posterior part, which denoted as the anterior part is broader than the posterior part. Furthermore, signs of morphogenesis occur as showing the development of larval limb buds (Figure 10E). After 3 hours, embryo within the embryonic cuticle develops the appendages (Figure 10F). After 3 hours 30 minutes, the anterior region

differentiates into a brown head and the posterior as the thoracic-abdominal region including further developing of appendages (Figure 10G). After 4 hours, the embryo develops the appendages (Figure 10H). After 4 hours 30 minutes, the embryo still develops appendages and not spreads out of the body. In addition, the posterior part is prolong more than four hours hydrated embryo (Figure 10I). After 5 hours, substantially changes occur in the embryo which is developing the appendages and beginning to appear the median naupliar eye (Figure 10J). After 5 hours 30 minutes, the embryo develops the antennae and mandibles (Figure 10K). After 6 hours, the embryo expands the antennae and mandibles outside the body including develops the swimming setae (Figure 10L). After 6 hours 30 minutes, the embryo develops the first and second antennae and mandibles as well as the anal pore, followed by emerging from cyst enclosed inside the embryonic cuticle (Figure 10M). Since after 7 hours of hydration, the fully formed naplius just hatches into a free swimming nauplius in the medium (Figure 10N-P).

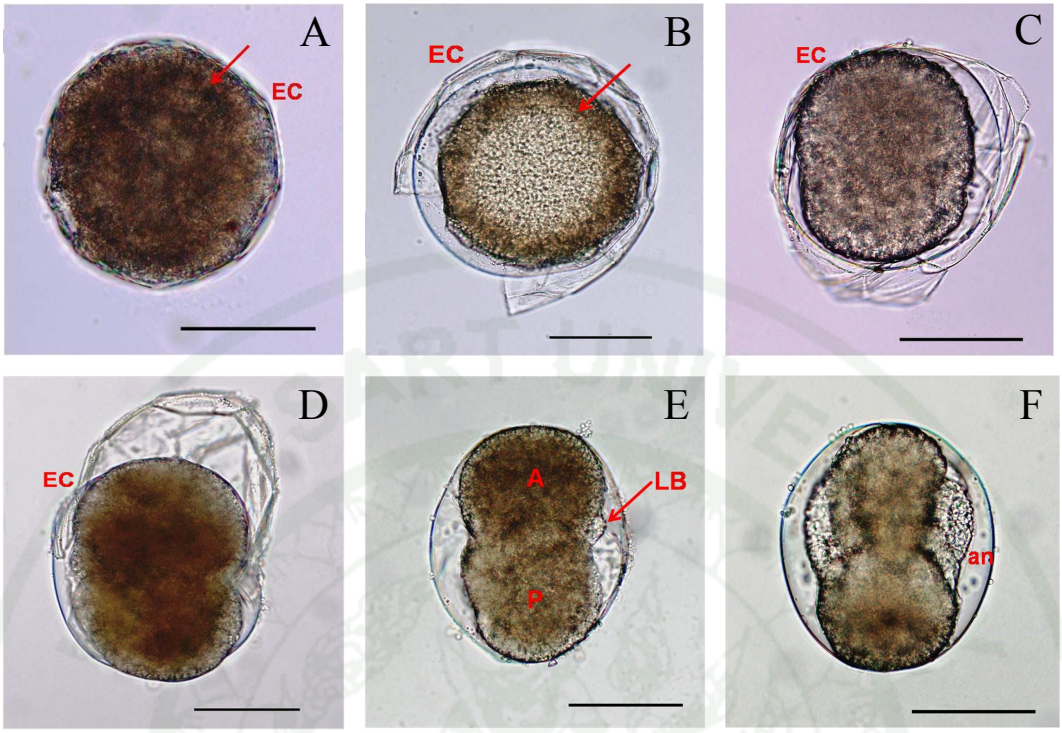

Figure 10 Embryonic development of *Streptocephalus sirindhornae* (Scale bar = 200 µm). **A,** After 30 min, cyst clearly shows prominent yolk granules. **B**, After 1 h, embryo is still undifferentiated and distinct appears yolk granules. **C**, After 1 h 30 min, yolk granules are gradually utilized (arrow). **D**, After 2 h, embryo divides into two hemi-halves. **E**, After 2 h 30 min, embryo differentiate into the anterior and posterior part. **F**, After 3 h, embryo develops the appendages. EC; embryonic cuticle, LB; limb bud, A; anterior part, P; posterior part, an; antennae.

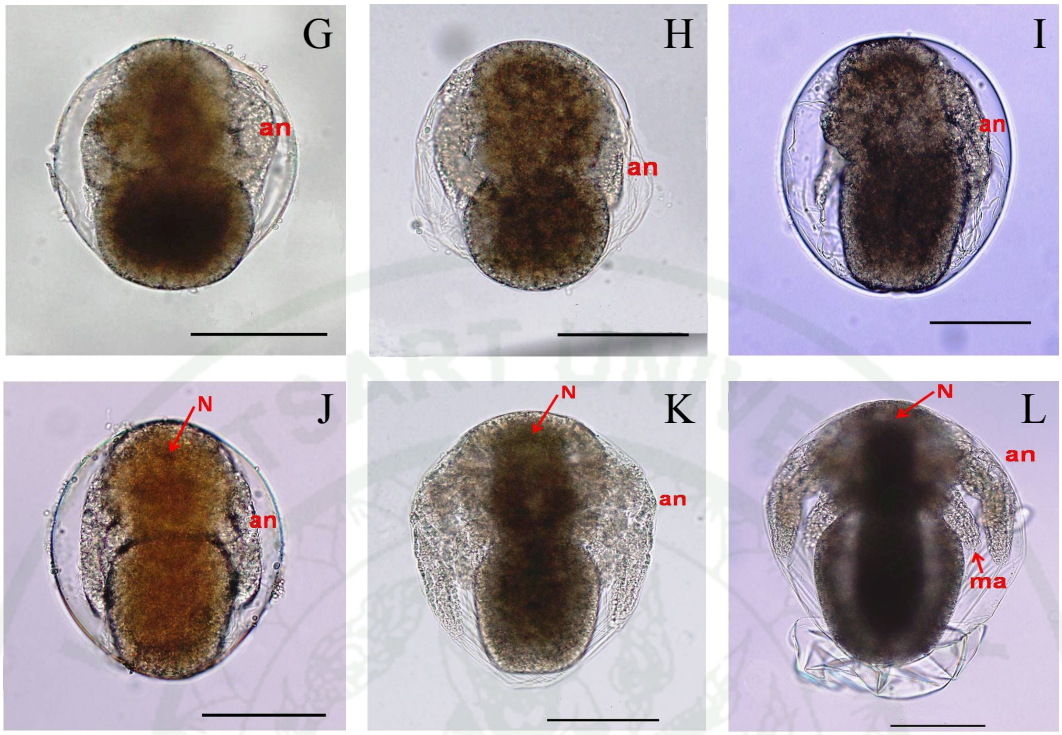

Figure 10 (Continued) **G**, After 3 h 30 min, the anterior region differentiates into a brown head and the posterior as the thoracic- abdominal region. **H**, After 4 h, the embryo develops the appendages. **I**, After 4 h 30 min, the embryo still develops appendages and not spreads out of the body.**J**, After 5 h, substantially develops the appendages and median naupliar eye. **K**, After 5 h 30 min, the embryo develops the antennae and mandibles. **L**, After 6 h, the embryo expands the antennae and mandibles outside the body. an; antennae, N; median naupliar eye and ma; mandibles.

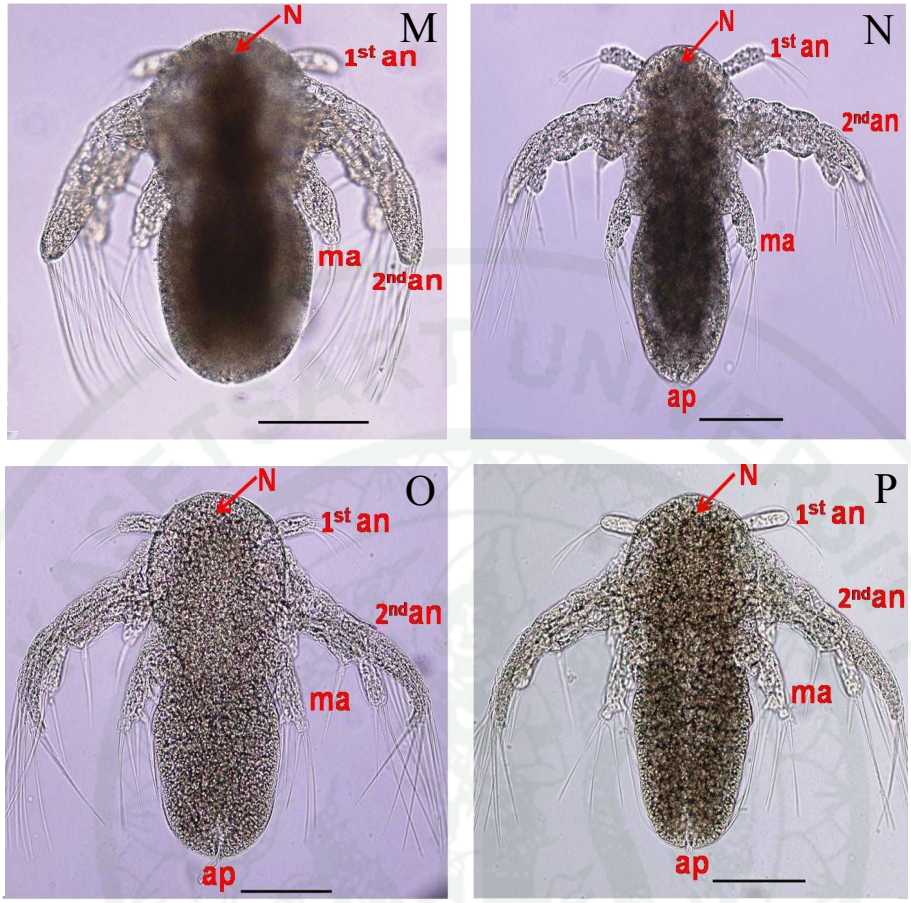

Figure 10 (Continued) **M**, After 6 h 30 min, the embryo develops the first and second antennae and mandibles as well as the anal pore. **N-P**, Since after 7 h of hydration, the fully formed naplius just hatches into a free swimming nauplius in the medium. N; median naupliar eye, 1st an; the first antennae, 2nd an; the second antennae, ma; mandibles, and ap; anal pore.

4. Preliminary explore the occurrence of stress proteins

The results of SDS-PAGE and Coomassie staining of proteins indicated that the large amount of proteins which are the component of fairy shrimp cysts. This study preliminary subjected to examine the stress proteins in the cysts of *B. thailandensis* and *S. sirindhornae*. The results are also shown as the bands which compare to molecular weight markers (Figure 9). There are three types of aliquots, homogenates, supernatants and pellets which can be determined the concentration of protein. The wide and dark bands indicate the amount of proteins. Almost of proteins are found in a major component in homogenates and supernatants fractions while slightly remained in pellets. As the results, *B. thailandensis* represents more clearly bands than *S. sirindhornae* especially in homogenates (h_1) and supernatants (s_1) while in pellets fraction (p_1) is slightly appearance.

This study aimed to predict the stress proteins (p26 and artemin) which occur in the cryptobiotic cysts in both of Thai fairy shrimps by observing the bands which appear less above 25 kDa. From the revised data, illustrate that p26 and artemin have the molecular weight masses at ~26 kDa, hence it can be slightly indicated that the cryptobiotic cysts of *B. thailandensis* and *S. sirindhornae* may be represented stress proteins, p26 and artemin.

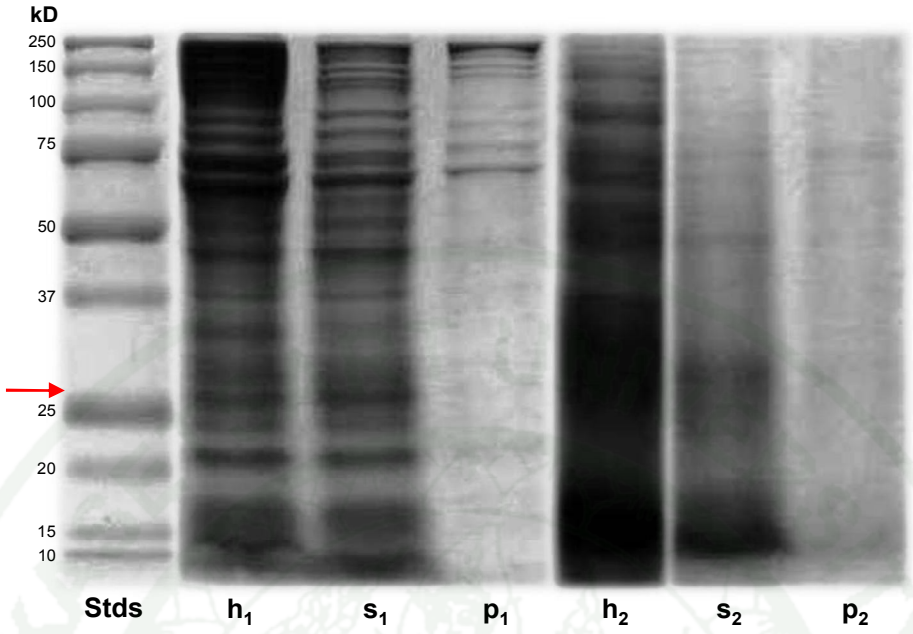

Figure 11 SDS-PAGE and Coomassie staining of protein in homogenats, supernatants and pellets of *Branchinella thailandensis* (h_1, s_1 and p_1) and *Streptocephalus sirindhornae* (h_2, s_2 and p_2). Equal volume of samples were applied per lane. The observe appearance bands in all samples (arrow line). Stds refer to molecular mass standards.

Discussion

As typical fairy shrimps and many anostracans produce cyst which the embryos are arrested at the gastrula stage, called as dormant embryos and also protected by the resistant shelled. Interestingly, the cryptobiotic cysts of fairy shrimps are the adaptive attributed in response to unpredictable environment, thus the life cycle are synchronized with a suitable environment for growth and reproduction (Munuswamy *et al*., 2009). Nowadays, the studies of Thai fairy shrimps have 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 (Boonmak *et al*., 2007). This study aimed at establishing the biology of two Thai fairy shrimps *Branchinella thailandensis* and *Streptocephalus sirindhornae* in terms of the structures of cyst and the embryonic developmental processes.

1. Scanning electron microscopy

Thai fairy shrimps *B. thailandensis* and *S. sirindhornae* exhibit internal fertilization and oviparity (Sanoamuang *et al*., 2002). Almost of mature eggs of both typical species have an external sculptured eggs shell with polygonal areas delimited by ridges. Cysts of *B. thailandensis* represent approximately 200 μm in diameter which larger than cysts of *S. sirindhornae* (150-180 μm). There are spherical shape with a regular pattern of pentagons and hexagons including appearance the ridges and depression regions. The external ornamentation is made up of pores and spiny projections. The embryo of *B. thailandensis* is protected from the external environment by the presence of a thick outer cortex (~25-30 μm), followed by a middle alveolar layer (~5-7 μm) and an innermost embryonic cuticle (~2-3 μm). According to Plodsomboon *et al*. (2012) reported that the structure of the sculptured shell of *B. thailandensis* belongs to the group without a sub-cortical space and composed an outer cortex (~20 μm), a middle smaller alveolar layer (~5 μm) and the innermost cuticular layer (~1μm). Cysts of *S. sirindhornae* show spherical shape with a regular pattern of variable polygons including pentagons and hexagons. There are also appearance the ridges with sloping sides and depression regions. Pores on sculptured surface of egg shell are prominent but disappear

the spiny projections which corresponding to Munuswamy *et al.* (2009), the cysts of *Streptocephalus dichotomus* show a regular pattern of polygons and also appear pores. Structural morphology of cross sectioned cyst of *S. sirindhornae* composes of indistinct three layers. The outermost is the outer cortex (~5µm) , the middle layer is the alveolar layer (~10-15 µm) which is characterized by an alveolar mesh and large lacunae, and also not demarcated with inner layer which appeared small variable size lacunae, and the innermost is the cuticular layer or embryonic cuticle (~2µm). Nearby, Previous study of Munuswamy *et al.* (2009), there are distinguishable three layers in the cryptobiotic cyst of *S. dichotomus*. The innermost enveloping the embryo is the cuticular layer, which appears in the light microscopy after decapsulated. The middle is alveolar layer characterized by an alveolar fibers connecting to inner layer, and the outermost is the thick cortex layer.

In this study, indicate that the difference features of cyst in term of ornamentation and internal structure between *B. thailandensis* and *S. sirindhornae*. *B. thailandensis* produces a larger cyst than *S. sirindhornae*, and also shows a regular of polygons including appears pores and spines, whereas *S. sirindhornae* produces cyst which disappears spiny projections. *B. thailandensis* exhibits the distinct three layers of shell which belongs to in a group without a sub-cortical space beneath the shell (Plodsomboon *et al*., 2012), while *S. sirindhornae* produces the indistinct three layers which classify into a group with a sub-cortical space and also occurs the large lacunae alveolar mesh (Munuswamy *et al*., 2009).

Fairy shrimps have also developed their biological characters to help them persist under unpredictable habitats and supports to withstand long periods of desiccation by producing the desiccated cysts. Moreover, 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 (Obregón-Barboza *et al*., 2002; Munuswamy *et al*., 2009).

The scanning electron micrographs of nauplii of the two species fairy shrimps revealed the characters of larval morphology. After incubation for 12 hours, nauplii

of *B. thailandensis* exists larger size than *S. sirindhornae,* approximately 400-450 µm and 250-300 µm, respectively. The differences recorded in size may be also evolved in filtering apparatus level relating to the different size prey selections and might reduce their food competition enabling them live together (Mura *et al*., 2003). Almost of the developmental process of the two species are similarity in the direction. However, *B. thailandensis* shows rapid growth and development more than *S. sirindhornae*, the anal pore is completed opening and also well developed caudal rami, while *S. sirindhornae* is still not appeared caudal rami in the distal posterior region. The study on the larval morphology will be conducted more understand in the developmental structure and will be used in a discussion of the basal phylogeny of the fairy shrimp.

2. Decapsulation and Hatching efficiency

The decapsulation technique is conducted for removing the outer part of the shell and chorion without affecting the viability of the embryos (Sorgeloos *et al*., 1977). There are two common methods (chlorine and chlorine/sodium hydroxide method) used to decapsulate cysts. For chlorine method, used household bleach (6% sodium hypochlorite solution) or industrial chlorine (11% sodium hypochlorite solution) for approximately 3-5 minutes, the alternative method is chlorine/sodium hydroxide method, which is used 50% sodium hydroxide in 11% sodium hypochlorite solution and should be performed in low temperature (4-7 ^{o}C) to prevent heat damage for about 3-5 minutes. This study was selected the chlorine method and subjected to examine the concentration of decapsulation chemical for removing the outer part of egg shell without the effect to embryonic development. Experimental data investigated that the decapsulated cysts of *B. thailandensis* and *S. sirindhornae* using 1% and 2% of sodium hypochlorite concentration represented the sameness hatching efficiency with controlled group or whole cysts. In this context, it is appropriate to mention that 2% of active ingredients hypochlorite solution can be used for releasing the external shell of eggs without affecting the viability of embryos, thus this appropriateness of concentration was conducted to clean the outer part of egg shell for observing the development of Thai fairy shrimps using light microscope. Nearby previous study of Sorgeloose *et al*. (1977), the optimum decapsulated concentration contained 2.12% of

active ingredients. Nevertheless, 4% and 6% of sodium hypochlorite as the high concentration and should be affected to embryonic mass. Based on the result of this study, the decapsulated cysts using 1% and 2% of sodium hypochlorite, showed no significant difference in the percentage hatchability with the whole cysts thus the decapsulation technique might not involve increasing in number of hatched egg. While, Tunsutapanich (1979) examined the comparison hatching assay of Artemia eggs between non-decapsulated eggs and those decapsulated with sodium hypochlorite, the results showed that decapsulated eggs have a higher hatching efficiency than those which are not decapsulated. Additionally, he introduced new chemical substances for use in the decapsulation process, such as lime (calcium oxide) and bleaching powder (calcium hypochlorite) which are cheaper than sodium hypochlorite and shown the same rate of hatchability. Although, the decapsulation might be not elevated the hatching efficiency, the process is improved for cleaning the outer part of egg shell. Furthermore, Sorgeloose *et al*. (1977) offered the advantages of the decapsulation procedure for practical use in laboratory work and aquaculture as 1) complete seperation of nauplii from the hatching debris since remained only the embryonic cuticle; 2) disinfection on external surface of cyst shells and 3) possible direct ingestion and digestion to decapsulated cyst by fish and crustacean larvae which reduced the deleterious effects. Notwithstanding, the appropriateness of kind and concentration of decapsulated substances is important because it can be affecting the viability of the embryos.

3. Embryonic development at difference timescales

Embryonic development of *B. thailandensis* and *S. sirindhornae* are observed ongoing hydration since 1 hour to 8 hours. The developmental process indicates the morphogenesis of developing embryo into the fully formed nauplius, which is a larval form with three pairs of appendages (antennules, antennae and mandibles) and a single median eye. *B. thailandensis* shows slightly rapid development more than *S. sirindhornae*. The sign of morphogenesis including larval appendicular buds and median naupliar eye occur within the early three hours in both Thai fairy shrimps. The developmental pattern of *B. thailandensis* is similar to *S. sirindhornae*.

After incubation, the embryo utilized yolk granules for developing, subsequently divided into two hemi-halves to differentiate into the anterior and posterior regions, by that the anterior part is broader than the posterior part. The anterior region develops into a brown head region and posterior as the thoracic and abdominal region. Furthermore, signs of morphogenesis occur as showing the development of larval lime buds. In addition, the appendages will be developing together with beginning to appear the median naupliar eye. The development completed to the naupliar stage, which the first and second antennae and mandibles develop as well as the anal pore can be observed, followed by hatching. Nauplius of *B. thailandensis* further develops and hatches into free swimming nauplius after 6 hours of incubation, while nauplius of *S. sirindhornae* retains approximately 7 hours for hatching into the medium. Our finding correspond with the SEM study which *B. thailandensis* deals the rapid development than *S. sirindhornae* and also according to the habitat observation by Dararat *et al*. (2011), which illustrated that *B. thailandensis* showed more rapid growth, largest body size, shortest hatching time, and the higher egg hatchability including generally inhabits in short-lasting pond, which have extreme fluctuations of water level and short period of inundation approximately 1-2 months. Therefore, Thai fairy shrimp *B. thailandensis* shows the biological characteristics that might have evolved as life mechanisms to survive under these environmental conditions. This observations also according to the work of Mura *et al*. (2003) who suggested that the population of fairy shrimp, *Chirocephalus diaphanus* Prévost, 1803 occurring in a shallow habitat presented rapid growth, precocious reproduction and short life cycle. On the other hand, *S. sirindhornae* was normally found in long –lasting ponds with water inundation approximately for 3-6 months. The life history of *S. sirindhornae* is characterized by slow growth, late reproduction, and long life span. The co-occurrence of fairy shrimps in Thailand has been reported. *B. thailandensis* co-occurs in several localities with the common species *S. sirindhornae*. *B. thailandensis* always emerged prior to *S. sirindhornae* and usually lived for 4-5 weeks (Sanoamuang *et al*., 2002). The two coexisting species generally inhabit in a group of ephemeral pond. The egg hatching of these two species seem to respond to the same hatching stimuli (Saengphan *et al*., 2005, 2009), since their nauplii can coexist but they can be separated by morphological size and their different maturation times. The study on life history information can also be useful for

the cultivation system of Thai fairy shrimp species. There are several biological attributes making them suitable for academic and commercial purposes.

4. Preliminary explore the occurrence of stress proteins

This study is particularly in term of molecular biology which preliminary explore the stress proteins of two Thai fairy shrimps. A goal of work was to explore the occurrence of stress proteins (p26 and artemin) in the cysts of *B. thailandensis* and *S. sirindhornae* using the simplest interpretation of our results by comparing with the previous works. From the revised data, indicated that p26 appear the molecular weight as 26 kDa (Clegg *et al*., 1999) and artemin as less above 26 kDa (Chen *et al*., 2003). Electrophoresis procedure in this work shows the protein bands near 26 kDa in both of *B. thailandensis* and *S. sirindhornae*, then can be slightly point that the cryptobiotic cyst of *B. thailandensis* and *S. sirindhornae* may be represented including p26 and artemin. From the experiment show the large amount of protein including p26 and artemin were occurred in homogenate and supernatant, while slightly appeared in pellet which corresponding to Munuswamy *et al*. (2009) that p26 were found in the homogenate, supernatant and pellet fraction, while artemin were strongly found in the homogenate and supernatant, but not in the pellet.

One of the most astonishing features of fairy shrimp encysted embryo is their ability to survive in the continuous stress conditions (Clegg, 1997). They are interrupted of development and extremely reduced metabolism (Drinkwater and Crowe, 1987; Drinkwater and Clegg 1991; Clegg, 1997). Whenever favorable conditions are presented such as sufficient water, suitable temperatures and adequate levels of molecular oxygen, the encysted embryos can be resumed development (Drinkwater and Clegg, 1991). Interestingly, diapauses development is the absence in both of DNA synthesis and cell division, until embryo emerges from the cyst (Nakanishi *et al*., 1962, 1963; Olson and Clegg 1978; Clegg and Conte, 1980), this unusual morphogenesis can also be interpreted as a significant adaptation because the embryo retains its resistant capabilities during post diapauses development and lose them after the embryo hatched. The importance remarkably of this point is

the occurrence of stress protein, p26 and artemin, which is the critical components of the adaptive repertoire that enables such as stability in the absence of DNA synthesis and cell division. According to Clegg *et al*. (1999) that point the correlation between the appearance of p26 in the diapauses-destined embryo, and the cessation of DNA synthesis and cell division during development, allows for the interesting possibility that p26 might be involved in the inhibition of DNA synthesis and cell division, either as part of the protection of DNA and other nuclear constituents. When activated encysted embryo are exposed to anoxia or thermal shock, about half of the p26 in the cytoplsam is translocated into the nucleus, and is reversed when the stress is removed, which corresponds to Derham and Harding (1999) that examined the translocation of p26 into nucleus in the heat shock or anoxia conditions *in vivo* and acidic pH *in vitro*. This evidence suggests that p26 also interacts with wide variety of nuclear/cytoplasmic translocation proteins which play an important role in the protection of nuclear lamins within the nuclear matrix (Willsie and Clegg, 2002), and responsible for the reduced transcription rate which is the reason for down-regulated RNA synthesis and protein synthesis. Beside p26, artemin is one of an important component which is mostly co-occurrence with p26 in the encysted embryo. Artemin exhibits RNA-binding ability and might be function as an RNA chaperone (Warner *et al*., 2004), responsible to Clegg *et al.* (1999) suggested that a plausible function for artemin is that it stabilizes or otherwise protects mRNA during the long term dormancy and severe stress. Thus, the occcurence of both stress protein, p26 and artemin, seem to be an important component of the adaptive repertoire of the encysted embryos that ensures their survival under the stress environmental conditions that they face in natural habitats.

CONCLUSION AND RECOMMENDATION

Conclusion

From the experimental results and discussion of this study, the conclusion can be drawn as follow:

1. The scanning electron microscopy investigate the characters of cysts that cysts of *B. thailandensis* and *S. sirindhornae* show spherical shape with a regular pattern of polygons including appearance of ridges with sloping sides and depression regions. Diameter of cyst is approximately 200 μm and 150-180 μm, respectively. Cyst of *B.thailandensis* appears pores and spiny projections, while spines are disappeared in *S. sirindhornae*. Structural morphology of cross-sectioned demonstrated three layers of egg shell in *B. thailandensis* (innermost embryonic cuticle, alveolar layer and outer cortex), indistinct three layers in *S. sirindhornae* (innermost embryonic cuticle, alveolar layer which is not demarcated with inner layer and outer cortex).

2. The scanning electron microscopy investigate the characters of nauplii that nauplii of *B. thailandensis* and *S. sirindhornae* exist approximately 400-450 μm and 250-300 μm in length. The three naupliar appendages, antennules, antennae and mandibles are well developed and functional as well as anal pore. The antennules are long, slender, unsegmented and bear three distal setae. The second antennae are large and biramous. The mandibles are inserted laterally on the body and consist of an enlarged coxa with the three segmented palps. The thoracic-abdominal part of *B. thailandensis* is seen as a cone shaped, whereas *S. sirindhornae* as egg like shaped. The trunk of *B. thailandensis* is weakly demarcated and almost occurred 4-5 segments of trunk lime buds, while *S. sirindhornae* is still not dematcated. The anal pore of *B. thailandensis* is completed opening and also well developed caudal rami in the distal posterior region but in *S. sirindhornae* is still not developed caudal rami in the posterior region.

3. The percentage hatchability of whole cysts, 1% and 2% decapsulated treatments, increased when increasing time through 6, 8 and 24 hours, respectively. There are no difference in hatching efficiency between whole cyst and 2% of 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.

4. Embryonic development of the two Thai fairy shrimps *B. thailandensis* and *S. sirindhornae* to the fully formed nauplius which is a larval form with three pairs of appendages (antennules, antennae and mandibles) and a single median eye under optimum conditions completed within 6 and 7 hours, respectively. *B. thailandensis* occurs the dramatic change around 2-4 hours by appearance the larval limb buds and median naupliar eye, whereas *S. sirindhornae* appears the spectacular change within 2.5-5 hours. After that, the embryo will be further developing into the fully formed nauplius.

5. The results of SDS-PAGE and Coomassie staining protocol predict the stress proteins (p26 and artemin) which occur in the cryptobiotic cysts in both of Thai fairy shrimps by observing the bands which appear less above 25 kDa. From the revised data, illustrate that p26 and artemin have the molecular weight masses at ~26 kDa, hence it can be slightly indicated that the cryptobiotic cysts of *B. thailandensis* and *S. sirindhornae* may be represented stress proteins, p26 and artemin.

Recommendation

This study is mainly focused on the structures of cyst and the embryonic development, thus the molecular work is an additive to investigate the attribute of cryptobiotic cyst. Hence, this work is the preliminary study on the molecular work which is predicted the protein with the revised data. Further studies will be adjusted the conditions and conducted more complexity procedure to illustrate the certainly kind of stress protein, either the techique of tryptic digestion and mass spectrometry or western immunoblotting.

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