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Evaluation of Morphological and Ultrastructural Changes of Black Tiger Shrimp (*Penaeus Monodon*) Spermatophore

Sumate Chomphuthawach[1], Teerasak Samosorn[2], Julaluk Juntaban[1], Bunlung Nuangsaeng[2], Rachanimuk Preechaphol[2], Vasin Yuvanatemiya[2], Subuntith Nimrat[3], Verapong Vuthiphandchai[4*]

[1]*Biological Science Program, Faculty of Science, Burapha University, Chonburi 20131, Thailand*
[2]*Facluty of Marine Technology, Burapha University Chanthaburi Campus, Chanthaburi 22170, Thailand*
[3]*Department of Microbiology and Environmental Science Program, Faculty of Science, Burapha University, Chonburi 20131, Thailand*
[4]*Department of Aquatic Science, Faculty of Science, Burapha University, Chonburi 20131, Thailand*

***Abstract**: The objectives of this research were to determine the morphological change of Penaeus monodon spermatophore and evaluate differentiation of sperm cell using transmission electron microscopy (TEM), and abnormality of sperm cell in male shrimp broodstock aged 8, 11, 14, 17 and 20 months. Results showed that 8 month-old shrimp had the appearance of spermatophore morphology to transparent (T) color, and did not appear the production of sperm mass inside spermatophore. Obviously, current results also showed that the duration of sperm maturation of P. monodon spermatophore was observed in shrimp at the age between 14 and 17 months with the presence of opaque white (OW) color, high condensation of chromatin and electron material, and low level of abnormal sperm. Exceptionally, age of shrimp selected at 11 months, although characteristic of spermatophore was presented to be OW color, sperm quality in this period was low showing high level of abnormal sperm as well as decondensation of heterochromatin and electron material inside sperm cell. However, at the end of experiment, there was the decrease in spermatophore quality appeared in selection at 20 month-old shrimp, forming of blackened spermatophore color, as a result of decrease of normal sperm and chromatin and electron decondensation was observed in this study.*

***Keywords**: Penaeus monodon, ultrastructure, morphology, spermatophore, abnormal sperm*

I. Introduction

In the last decade, aquaculture industry plays a major role for national income of Thailand due to high production rate. One of the most important species for demand of the world market is penaeid shrimp. Black tiger shrimp (*P. monodon*) was widely cultured in the Eastern and Southern parts of Thailand with production of more than 400,000 tons/year [1, 2]. However, in the last several years, there was a disease outbreak including infection of contaminated pathogenic organism of bacteria (*Vibrio harveyi*) [3] and Pseudomonas [4], especially infection of viral pathogen such as Laem-Singh Virus (LSNV), Taura Syndrome Virus (TSV), White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV) or Monodon Baculo Virus (MBV) [5, 6, 7, 8]. As a result, there was a rapid decline in the production of *P. monodon* lower than 100,000 tons in 2005 [9, 10]. These pathogenic infections were considered to be the most limiting factor for production of *P. monodon* industry, which directly affected on the shortage of good quality broodstock for larvae production.

In the recent years, broodstock domestication became an alternative way to minimize disease transmission from broodstock to larva and facilitate selective breeding program under the hatchery operation [11, 12]. Although domestication program of *P. monodon* has been initially developed since 2007 to facilitate the breeding program, a decrease in spawning and fertilizing rates after artificial insemination (AI) has also been prominently observed compared with the use of wild stock [1, 12, 13, 14]. Early researches have also reported low percentage of hatched eggs using domesticated broodstock of *P. monodon* inseminating under hatchery condition [13, 14]. However, Menasveta [13] suggested that the major problem for the failure of fry production has been associated with decrease of sperm quality inside spermatophore as a result of the absence of final sexual maturation of sperm inside spermatophore [15]. Some studies confirmed that the development of reproductive tract of whiteleg shrimp (*Litopenaeus vannamei*) was dependent on the appropriate period of age and weight of shrimp to produce sperm maturation [16, 17]. Moreover, evaluation of the change in color of spermatophore from different shrimp age is one of the most crucial parameters for assessment of readiness of reproductive tract and change in sperm quality. Male *L. vannamei* with older age had a decrease quality of spermatophore due to degradation process of cellular matrix contained in outer-layer of spermatophore [15, 18]. Jiang et al. [19] reported that *P. monodon* reared in the earthen pond at ages from 157 days to 258 days had an increase of sperm count from $0.63x10^6$ cell/ml to $12.41x10^6$ cell/ml, respectively. Additionally, there were some

researchers reporting about the relationship of spermatophore development and shrimp age. For example, Cebellos-Vazquez et al. [16] reported that the maturation of *L. vannamei* was obtained from shrimp cultured for 12 months, producing $4.57x10^6$ cell/ml for sperm count, 68.2% for normal sperm and 0.093 g/spermatophore for spermatophore weight. Crocos and Coman [20] and Coman and Crocos [21] have also indicated that sexual maturity period for the reproductive system of *Penaeus semisulcatus* shrimp was observed at a late adult phase (>12 months), and then presented with deterioration of spermatophore quality over the cultured time. At the present, information of sperm quality determined based on the change of spermatophore characteristic in relation to the male age of pond-reared shrimp has been limited. Hence, better understanding of appropriate period for sexual maturation of *P. monodon* shrimp could be beneficial for enhancement the fertilization and hatching rates, and would be an important baseline information for performance of reliable stocks prior to selective breeding program via AI process.

The aim of this research was to investigate the change in color of *P. monodon* spermatophore under visual observation and the change in ultrastructural characteristic of sperm cell using TEM. Abnormality of sperm cell was also evaluated using light microscope (LM) by the use of male shrimp broodstock from different ages (8, 11, 14, 17 and 20 months) under pond-reared condition.

II. Materials and Methods

2.1 Shrimp maintenance

The stage of PL 15 *P. monodon* obtained from Shrimp Genetic Improvement Center (SGIC), Suratthani Province, were cultured in the cement tank (wide x length x high: 4 x 5 x1.2 m) with a density of 60 shrimp/m^3 at the Marine Technology Research Center (MTRC), Burapha University Chanthaburi Campus. Water qualities were measured at every day in the morning prior to feeding including of salinity level (29-30 ppt), pH (7.5-8.5), dissolved oxygen (DO) (>4mg/L) and nitrite (NH_3) (<0.1 ppm), then followed by water exchange about 50% per day from filtered sea water tank. After 30 days of culture with about $3x10^6$ cell/ml *Chaetoceros* spp., shrimps were fed with pellets food with 35% protein level allocating to 3-5% of shrimp body weight at four times a day. Vitamin E was supplemented during the culture once a week by mixing with pellets at a ratio of 5 g vitamin E/ 1 kg pellets.

2.2 Selection of shrimp spermatophore

In order to obtain *P. monodon* spermatophore, shrimp were unfed for 10 hrs before the capture. Male shrimp were randomly selected for each time in a number of 20 shrimp during the age of 8, 11, 14, 17 and 20 months using a large hand-net. After capture, shrimp were immediately retained in Styrofoam box supplied with cultured sea water and aerated by the electric pump during selection of spermatophore. Shrimp spermatophores were manually obtained by gentle pressing around coxae area of the fifth walking legs, and afterward a pair of extruded spermatophore was collected using the sterile forceps. Extruded spermatophores were immediately placed on Petri dish on ice (~4ºC) about 8-10 spermatophore/ Petri dish to reduce the morphological change of spermatophore from dehydration process. After selection of spermatophore for each sampling time, all male shrimps were removed to the other tank early prepared to reduce variation for next selection.

2.3 Spermatophore determination

Newly selected spermatophores were characterized by photographing of extruded spermatophore placed on sterile Petri dish using digital camera (Sony, Model NO.DSC-H2, Japan). Evaluation of morphological change of selected spermatophore was done by visual observation on the alteration of the color of spermatophore wall after each selection. Consideration of spermatophore color was separated into 3 levels including transparent (T), opaque white (OW), and melanized (M). Semithin section technique was used to evaluate abnormal sperm under LM (described next section). Determination the abnormality of sperm cell was divided into 2 levels of high and low abnormal sperm by observing characteristic the presence of missing or bending spike.

2.4 LM and TEM preparations

Freshly stripped spermatophores were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer solution (PBS) at 4ºC temperature (on ice), and cut in small pieces before washing and shaking (30 rpm/min) three times for 5 min each with 0.1 M PBS. Washed specimens were again fixed with 2% osmium tetroxide (OsO_4) in 0.1 PBS for 2 hours. After OsO_4 fixation, specimens were immediately dehydrated with ethyl alcohol for 30 min at a concentration of 70, 80, 90, 95 and 100% respectively, followed by infiltration of specimens into flat mold containing propylene oxide (PO) and araldite 502 resin at 30 rpm/min for 12 hours. Infiltrated specimens were incubated in incubator at 60ºC for 24 hours and localized specific position of sperm cells were stained with 1% methelene blue. Localized specimens were slightly cut at 1 µm thickness using semi-thin section technique prior to viewing under LM at 100x magnification. For TEM preparation, specimens obtained

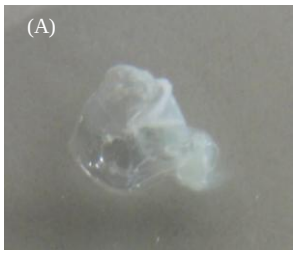
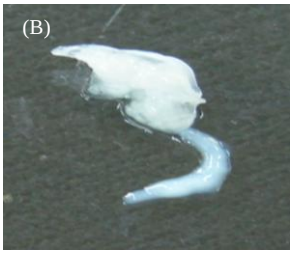
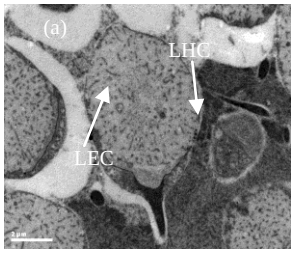
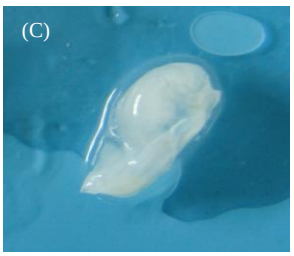
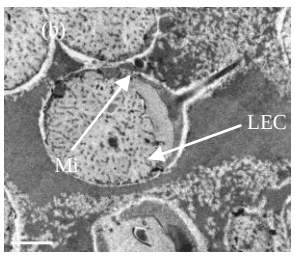
with a semi-thin technique were again sectioned at 50-70 nm thickness, and then stained with uranyl acetate (UA) and 0.1% lead citrate before viewing under TEM at 75 kV.

III. Results

3.1 Change in morphological and ultrastructural characteristic of *P. monodon* spermatophore

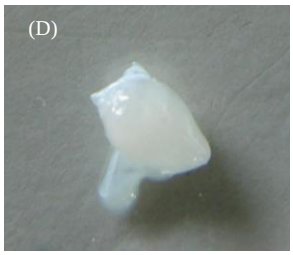
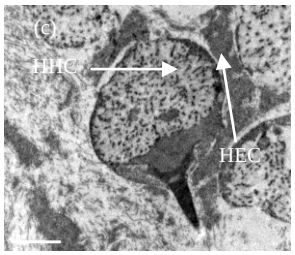
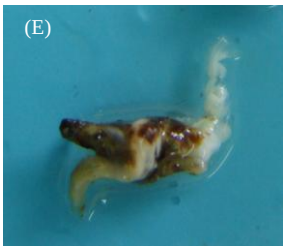
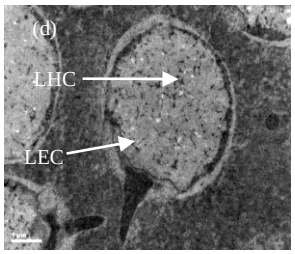
The change of spermatophore color and differentiation of sperm ultrastructure of *P. monodon* shrimp selected at the age of 8, 11, 14, 17 and 20 months were shown in Table 1. There was no observed sperm mass inside spermatophore of shrimp aged 8 months (Table 1A), appearing color of T spermatophore. For the shrimp aged of 11, 14 and 17 months, spermatophore was observed with the presence of OW color (Table 1B, C, D). Ultrastructeral changes of sperm cell from 11 month-old shrimp showed low condensation of heterochromatin and membranous envelope throughout the cell (Table 1B). Shrimp aged between 14 and 17 months showed differentiation of sperm cell with the first appearance of mitochondria (Mi) organelle locating at the rim region of the cell, especially heterochromatin material and membranous envelope were highly condensed at 17 month-old shrimp (Table 1C, D). At the end of the experiment, shrimp at 20 month-old had an appearance of spermatophore with M color (Table 1E), followed by a decrease in heterochromatin materials throughout the sperm cell as well as partial absence of electron condensation at the membranous envelope (Table 1D) compared with that of other shrimp age.

Table 1 The change of spermatophore characteristic in color and cellular ultrastructure of spermatophore selected from *P. monodon* shrimp at different ages.

Age (month)	Spermatophore characteristics	Spermatophore color	Ultrastructural differentiation (TEM)
8		T	NSM
11		OW	
14		OW	

An abbreviated letters referred: T = transparent; OW = opaque white; M = melanized; LHC = low heterochromatin condensation; LEC = low electron condensation; Mi = mitochondria; HHC = high heterochromatin condensation; HEC = high electron condensation; NSM = no sperm mass

Table 1 continued

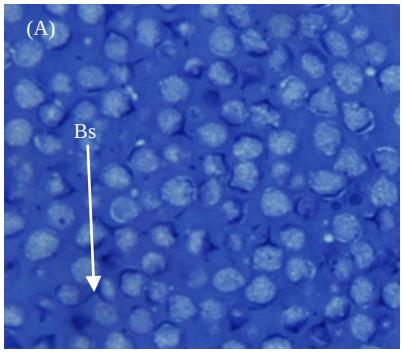
Age (month)	Spermatophore characteristics	Spermatophore color	Ultrastructural differentiation (TEM)
17		OW	
20		M	

An abbreviated letters referred: T = transparent; OW = opaque white; M = melanized; LHC = low heterochromatin condensation; LEC = low electron condensation; Mi = mitochondria; HHC = high heterochromatin condensation; HEC = high electron condensation; NSM = no sperm mass

3.2 Evaluation of abnormality of *P. monodon* sperm

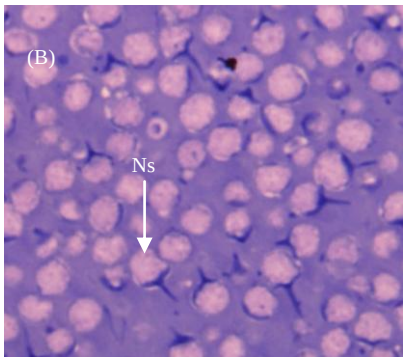
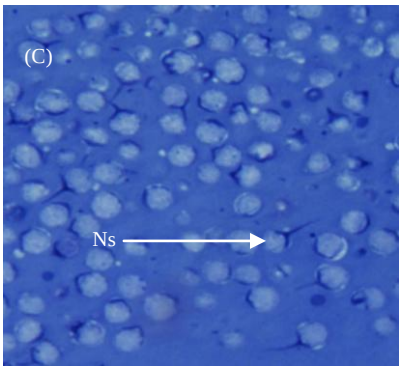
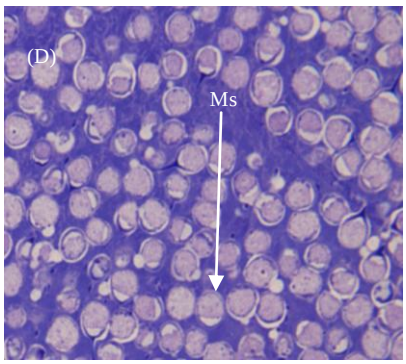
The change in characteristic of *P. monodon* sperm of shrimp collected at 11, 14, 17 and 20 months was observed under LM (Table 2). Shrimp aged at 8 months did not have sperm mass inside spermatophore. Shrimp at 11 month-old shrimp had high level of abnormal sperm cell (HAS) showing bending sperm spike more than 50% (Table 2A). In contrast, shrimp aged 14 and 17 months exhibited low level of sperm abnormality (LAS) less than 50% and contained normal characteristics of sperm cell (Table 2B, C). However, at the end of experiment, there was an appearance of missing sperm spike observed in the 20-month old shrimp with high sperm abnormality (more than 90%) (Table 2 D).

Table 2 An abnormality of sperm cell selected from *P. monodon* shrimp at different ages.

Age (month)	Sperm characteristic (LM)	Levels of abnormal sperm
8	NSM	NSM
11		HAS

An abbreviated letters referred: Bs = bending spike; Ns = normal sperm; Ms = missing spike; NSM = no sperm mass; HAS = high abnormal sperm (>50%); LAS = low abnormal sperm (<50%)

Table 2 continued

Age (month)	Sperm characteristic (LM)	Levels of abnormal sperm
14		LAS
17		LAS
20		HAS

An abbreviated letters referred: Bs = bending spike; Ns = normal sperm; Ms = missing spike; NSM = no sperm mass; HAS = high abnormal sperm (>50%); LAS = low abnormal sperm (<50%)

IV. Discussion

The change in color of *P. monodon* spermatophores obtained from different age (8, 11, 14, 17 and 20 months) was directly related with abnormality and differentiation of sperm cell. The presence of transparent color of spermatophore and absence of sperm mass inside spermatophore of 8-month old shrimp indicated that the readiness for development of spermatophore in reproductive tract was not fully developed. In addition, the presence in T color distributed throughout spermatophore was likely to be the clear fluid substances produced from the region of proximal vas deference [22]. Erken et al. [23] and Braga et al. [24] reported that the presence of clear gelatinous substrates contained inside spermatophore layer had important properties for adhesive protection of pathogenic bacteria from the environment condition as well as for maintaining sperm cell viability inside spermatophore layer. However, this layer of spermatophore was previously believed to be associated with production of hyaluronic acid substance that was produced by the secretary cells of epithelia tissue to protect viral and bacterial infection during living of sperm in spermatophore [22]. The presence of spermatophore

characteristic with OW color, incidence of LAS, formation of Mi organelle and high condensations of heterochromatin and electron materials, this indicated that the reproductive tract of *P. monodon* shrimp was fully developed at this period. This result was similar to the works reported by Pongtippatee et al. [25] and Poljaroen et al. [26] who suggested that condensed chromatin inside main body (MB) of matured sperm of *P. monodon* was essential in some mechanisms related with the reaction of depolymerization process of sperm spike region or microfilament formation of cytoskeleton structure occurred during the acrosomal reaction (AR). In contrast, shrimp at 11month-old had high level of abnormal sperm with bending of sperm spike (Table 2A). This was in agreement with Jiang et al. [19] who indicated that male *P. monodon* shrimp cultured in the earthen pond started to form sperm mass inside spermatophore when shrimp aged approximately 4.5 months with percentage of abnormal sperm about 92.2% and afterward decreased (<75%) when shrimp aged 8.5 months. The appearance of OW spermatophore and high level of normal sperm of 11 month-old shrimp of the present study indicated that the development of reproductive tract started to have the formation of sperm mass prior to transportation to terminal ampoule. Additionally, the present of low condensation in both of electron material at the membranous envelope and heterochromatin proteins around cytoplasm of the cell indicated the incidence of abnormal sperm at this period (11 months). Decondensation of heterochromatin and electron material was believed to be associated with mechanism of protein synthesis modified to generate intracellular activities as in catabolism or anabolism process [25]. Quality of spermatophore was reported to be dependent on the change of spermatophore color and percentage of abnormal sperm [18]. Crocos and Coman [20] and Coman and Crocos [21] reported that the sexual maturation of *P. semisulcatus* was observed to form light white color of spermatophore in shrimp aged more than 12 months, and then the quality of spermatophore deteriorated over time. Another research studied by Primavera and Quinitio [27] who suggested that unsuccessful mating using natural broodstock of *P. monodon* shrimp has been directly related with the shortage of good quality spermatophore based on appearance of light white spermatophore. Cebolles-Vazquez et al. [16] reported that development of *L. vannamei* spermatophore with formation of white color occurred in shrimp aged between 10 and 12 months, but the percentage of abnormal sperm was gradually decreased from 25.6% to 17.4%.

Shrimp aged between 14-17 months exhibited sexual maturation of the reproductive tract due to the presence of OW color of spermatophore. Despite the absence of evaluation of sperm count and percentage of viable sperm of the present study, morphological and ultrastructeral change of sperm inside spermatophore could be an important indicator for selection of good quality spermatophore prior to AI operation. Accordingly, Goimier et al. [28] emphasized that high sperm count (>6.8x 10^6 cells/ spermatophore) of *Litopenaeus sertiferus* after feeding with 45% protein resulted in better fertilization and hatching rate after natural matting. Similarly, Cebellos-Vazquez et al., [16] reported that *L. vannmei* cultured for 12 months had spermatophore with white color that provided high percentage of normal sperm (68.2±2.7%) although normal sperm decreased (<40%) after 15 months of culture. Deterioration of spermatophore with appearance of M color (blackening spermatophore) at the end of experiment for 20 month-old shrimp of the present study was related with melanization of spermatophore that was directly involved with an increase in during the cultured period. Peraz-Velazquez et al. [29] studied about the effect of water temperature (26, 29 and 32ºC) number of sperm of *L. vanamei* broodstock maintained in the circular tank and found that high water temperature of 32ºC could dramatically reduce an average of sperm count from 18.6±1.75 million/ml (26ºC) to 0.1±0.05 million/ml (32ºC). An increase of water temperature of the present study from 29.4ºC (beginning of experiment) to 33.7ºC (end of experiment) may led to deterioration of spermatophore and sperm quality, showing melanized spermatophore and missing and bending spike of sperm. However, incidence of blackening spermatophore was previously reported to be associated with the expression of SERPIN8 (serine proteinase inhibitor) gene leading to activation of prophenoloxidase (proPO) system to produce melanin by polymerization process [30, 31]. Therefore, the presence of blackening spermatophore (melanization) at the end of experiment indicated that the response to the immunological system for suppressing melanin pigmentation in older male shrimp may less sensitive than young male shrimp.

V. Conclusion

In conclusion, present results demonstrated the change in characteristic of *P. monodon* spermatophore with the presence to T color in shrimp aged 8 months that did not have sperm mass production inside spermatophore. Shrimp aged 14 and 17 months exhibited sexual maturity of reproductive tract with the formation of good quality spermatophore of OW color, LAS occurrence and complete intracellular composition of Mi organelle, chromatin and electron condensation. An incidence of spermatophore melanization and high sperm abnormality of missing spike of 20 month-old shrimp was related with an increase of water temperature. This work represented the first report on correlation of sperm morphology and ultrastructure of *P. monodon* broodstock raised in captivity, serving as baseline information for selection of good quality spermatophore from optimal period to enhance fertilization capacity via AI process.

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