TRIPLOIDY INDUCTION USING COLD SHOCK TREATMENT IN BANANA SHRIMP, PENAEUS MERGUIENSIS (DE MAN, 1888)

[1*]Hidayah, M., [1]Ikhwanuddin, M., [2]Shahreza, M.S., [1]Abol - Munafi, A.B., & [3]Mohd Fariddudin, O.

[1]Institute of Tropical Aquaculture,
Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu.
[2] Faculty of Agrotechnology and Food Science,
University Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu.
[3] Fisheries Research Institute Pulau Sayak, 08500 Kota Kuala Muda, Kedah, Malaysia.

ABSTRACT. *A study was conducted to determine the optimum parameter to produce triploid in banana shrimp, Penaeus merguiensis using cold shock treatment. Three different temperatures were introduced to the shrimp eggs after 10 minutes of spawning which are 10°C, 15°C and 20°C with 3 different times of exposure (10, 15 and 20 minutes) for each temperature. Three hours after treatment, 20 eggs from each treatment were collected for chromosome analysis. Chromosome numbers were analyzed using Video Karyo Test software version 3.1. It was found that 15°C with duration of 10, 15 and 20 minutes induced 100% triploid (3n). The metaphase chromosome number ranged from 118 -154 with the mode of 132 chromosome. Treatment conducted at 10°C and 20°C for the same duration of time produced diploid, (2n) and the metaphase chromosome number ranged from 86 - 88 with the mode of 88 chromosome. The findings of this study indicate that cold shock treatment at 15°C appears to be the optimum temperature for production of triploid in banana shrimp.*

KEYWORDS. Banana shrimp, *penaeus merguiensis*, cold shock treatment, triploid.

INTRODUCTION

The application of biotechnology in penaeid prawn aquaculture is increasingly important (Benzie, 1998). In terms of value, shrimps are the most important seafood products traded globally with production from aquaculture estimated at 3.1 million metric tonnes valued at US$12 billion in 2008 (Shrimp News International, 2009; Sellars *et al*., 2010). *P. merguiensis* is the commercially important marine shrimp species in the Indo-Pacific region including Australia, New Guinea, Indonesia, Philippines and to a lesser extent in Malaysia, India, Pakistan and the Arabian Gulf (Phongdara *et al*., 1999). The slow growth of *P. merguiensis* in pond grow out culture compared to *Penaeus monodon* has been the problem in aquaculturing this species. More male shrimp being produced than females and male shrimp being smaller than females results in less product in the market (Choo, 1984). Nowadays monosex culture has become a common practice in aquaculture. Attempts have been made to apply this aquatechnology to crustacean culture since male and female crustaceans differ in growth rates, behavioral pattern and husbandary needs (Aflalo *et al.*, 2006). Many previous studies have been done to confer sterility in invertebrates and fish including polyploidy, interspecies hybridization, hormonal treatment, irradiation and regulation of gene expression. Polyploidy is the only known technique that can achieve the dual outcomes of reproductive sterility for genetic protection and skewing sex ratios. Although not yet commercialized for shrimp, several examples of polyploid technology are already adopted as part of standard commercial farming practices (Sellar *et al*., 2010). Triploidy induction is a technique that allows genetic manipulation of chromosome number to control reproduction and to manipulate the potential to create faster growing animals (Harrell *et al.*, 1998). Induction of triploidy in shrimp is useful in increasing the size of juvenile and matured shrimp to suit commercial demand (Brake *et al*., 2004; Xiang *et al*., 2006). Sterility may be accompanied by better growth after sexual maturation because energy is not diverted to reproduction (Dumas & Ramos, 1999). Generally triploids are produced by shocking eggs

shortly after fertilization to prevent the second polar body (SPB) from leaving the egg. This produces a fertilized egg that has a haploid (n) egg nucleus, a haploid sperm nucleus and a haploid SPB nucleus. These three haploid nuclei fuse to form a triploid zygote nucleus (Tave, 1990). Several reports have been written on the induction of triploidy and gynogenesis in marine species including sparids, moronids and flatfishes (Shelton, 2006). High success rate of triploid and gynogenetic induction can be easily achieved depending upon optimization (Felip *et al*., 2001). Triploid induction techniques have been developed for a number of penaeid species (Xiang *et al*., 2006; Coman *et al*., 2008). In shrimp, triploidy is the most successful method for inducing sterility (Li *et al*., 1999; Norris *et al*., 2005; Sellars *et al*., 2006; Xiang *et al*., 2006; Coman *et al*., 2008). According to Xiang *et al.* (1992) ploidy manipulation is used on a commercial scale for *Penaeus chinensis*. No definitive reports or detailed studies have been completed on triploidy induction in other penaeid shrimps (Coman *et al*., 2008) especially for *P. merguiensis* shrimp for the purpose of commercial scale production. Therefore, the objectives of this study are to induce triploidy using cold shock treatments and to identify the optimum parameters for successful cold shock treatments in banana shrimp, *P. merguiensis.*

MATERIALS AND METHODS

Broodstock, spawning and egg collection

Wild gravid females of *P. merguiensis* were caught from coastal water of Pulau Sayak, Kedah (lat: 5° 40' 0"N and long : 100° 19' 60"E) and all the shrimps were transported back to the marine hatchery of Institute of Tropical Aquaculture (AKUATROP), University Malaysia Terengganu (UMT) for spawning. Gravid female shrimps were transferred to the breeding tank with stocking density of 360 L /individual. The gravid females were kept in sea water at 30 ppt and the spawning behaviour was constantly observed until the shrimp spawned. After spawning, the eggs were immediately collected using a modified sieve (60µm mesh size) and were used for triploidy induction.

Induction of triploidization using cold shock

Tanks of three different temperatures,10°C, 15°C and 20°C, were prepared using marine ice and one control tank (28°C) was prepared. A thermometer was used to measure the temperature for each treatment. After 10 minutes spawning, the collected eggs were transferred to the modified sieve for each treatment. The time of application of the shock was determined after previous microscopic observation of the extrusion of polar bodies. They are expelled at 8 and 15 min after spawning. Survival was verified at the blastula-gastrula stage by microscopic observation. After treatment, the eggs were transferred to a 10 L aquarium and for each treatment 20 eggs were collected and treated with 0.05% colchicine for about 60 minutes to be used for chromosome analysis.

Chromosome triploidy determination

Chromosome staining

The protocol developed by Dumas and Ramos (1999) was applied with some modifications. The eggs in colchicine solution were transferred to a 1.5 ml tube and centrifuged at 1000 rpm for 5 min. After centrifugation, the eggs tissue was transferred onto the glass slides and then treated with hypotonic solution of 0.1M KCl for 25 min. Then, the tissues were fixed in 3:1 Carnoy's fluid (3 parts methanol and 1 part glacial acetic acid) for 1hour with 12 minutes for every change of the fluid. After fixation, tissue was treated with 50% acetic acid to submerge the tissue. The slides were air dried for 10 minutes. Next, the slide was broken by slight blow and stained with 15% Giemsa buffered solution (40 mol Na_2HPO_4 with 26.6 mol $KHPO_4$) for 10 minutes. Slides then were air-dried for 5 minutes and samples were continually observed under microscopes.

Triploidy detection and identification

Chromosomes at the various metaphase stages were examined for each treatment and chromosome photos were taken using the Advance microscopes *Nikon eclipse* 80i. Data of chromosome triploidy were analysed using Video Karyo Test software (version 3.1). Chromosome pictures were detected using the analysis mode on the Video Karyo Test software and the actual chromosome numbers were counted and identified using the metaphase mode function.

DATA ANALYSIS

The actual chromosome number for each treatment at 10^0C, 15^0C and 20^0C for each exposure time at 10, 15 and 20 minutes was counted using Video Karyo Test software. The mean numbers of chromosomesin the pictures were counted and the success of triploid produced in each treatment was determined.

RESULTS

It was found that the highest number of metaphase chromosomes counted was between 118 to 154 at 15°C. Treatment conducted at 15°C with duration of 10, 15 and 20 minutes induced 100% triploidy (3n) with mode of 132 chromosomes (figure 1). Treatments at 10°C and 20°C for the same duration produced diploidy, (2n) with metaphase chromosome number between 86 and88 with mode 88 chromosome (figure 2). Data of the triploid successes for each treatment and the actual number of chromosomes counted are given in Table 1.

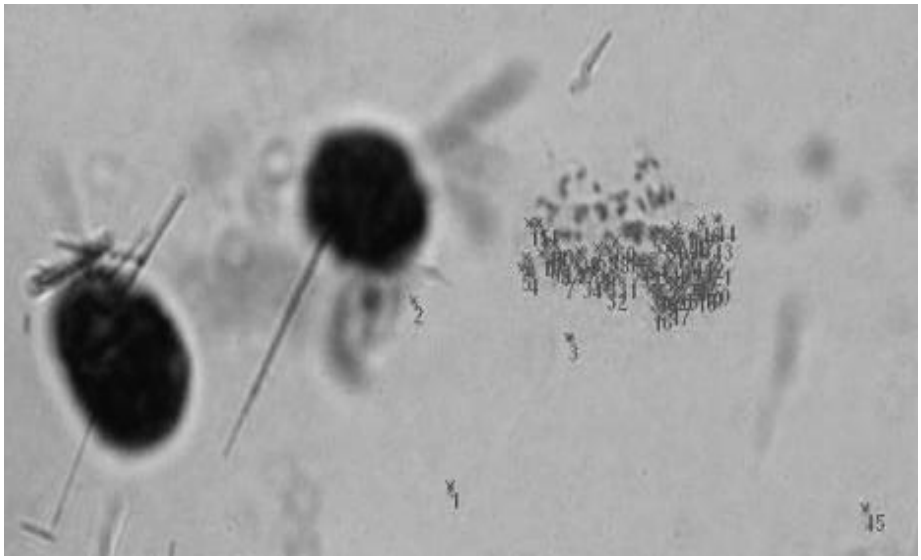
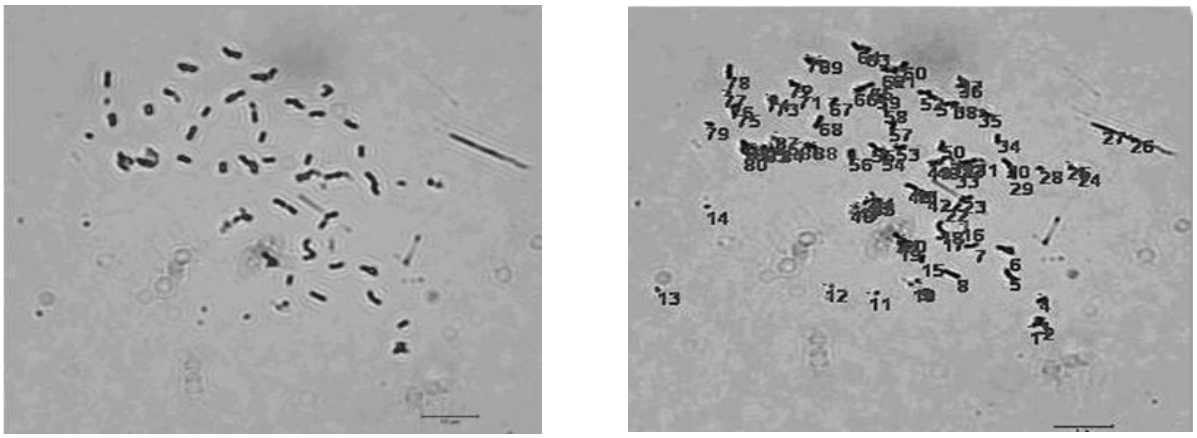


Figure 1. Picture of chromosomes during counting using Video Karyo Test software at 15°C and 15mins duration shock treatment (100x magnifications).



(a) (b)

Figure 2. Chromosomes pictured using Video Karyo Test software before (a) and after (b) the treatment at 10°C and 15mins duration shock (100x magnification).

Table 1. Number of chromosomes in *P. merguiensis* shrimp eggs in each cold shock treatment with the success of triploid induction(n=20).

Optimize Temperature (°C)	Durationof shock time (min)	Produce diploid, (2n) or triploid, (3n) of fertilized egg	Range for no.of chromosomes counted	Mean ±SD for No.of chromosomes counted
10	10	2n	88	88±0.00
10	15	2n	88	88±0.00
10	20	2n	88	88±0.00
15	10	3n	118-154	132.66±5.94
15	15	3n	132-142	134.09±4.02
15	20	3n	132-136	132.19±0.87
20	10	2n	88	88±0.00
20	15	2n	86-88	87.90±0.43
20	20	2n	88	88±0.00

DISCUSSION

Successful triploids were produced due to the accurate time to induce shock 10 minutes after the spawning before the extrusion of second polar body. Study done by Dumas and Ramos (1999) found that the polar bodies are expelled at 8 to 15 min after the spawning and at 16 min were already extruded out of the eggs when it is possible to induce triploid. In the present study, the chromosomes in metaphase stages were viable and could be observed 3 hours after the spawning. According to Xiang *et al.* (1994) obtaining chromosomal metaphases from embryos and larvae is helpful for early successful chromosomal manipulation in marine shrimps. From the results obtained, it was identified that shock at 15°C for 10 minutes, 15 minutes, and 20 minutes could produce triploid individuals because of the potential of the temperature to prevent the second polar body (PBII) from extruding out of the egg which can shock the shrimp eggs and induce triploidy. Otherwise, shock at 10°C and 20°C for the same duration time could not produce triploid organisms but produced normal number of chromosome (88 chromosomes). Previous study on *P. merguiensis* showed that it has 44 pairs of chromosomes (2n=88) for the diploid individual

(Xiang, *et al*., 1994). The reason for the triploidization at 15°C could be the local temperature at around 27°C to 28°C which means that 15°C can shock the shrimp eggs and induce triploidy. However, it is different with *Litopenaeus vannamei* that lives in tropical marine habitats with temperatures normally just above 20°C throughout the year (FAO, 2011). Triploidy successfully induced in *Litopenaeus vannamei* at 10°C shock for 10, 15 and 20 minutes at10 and 12 minutes after spawning (Dumas & Ramos, 1999) differs from the temperature suitable to produce triploidy in *P. mergueinsis*.Otherwise, 84.5% triploid was found in the nauplius stages of *Fenneropenaeus chinensis* shrimp using temperature shock treatment at 18°C (Zhang, 2003) which also differs from the temperature suitable to induce triploid with *P. merguiensis*. According to Aloise and Francisco (2010), cold thermal shock was more efficient than hot ones to induce triploid on *Litopenaeus. vannamei*. This shows that the cold shock treatment is the most suitable method to induce triploid in *P. merguiensis*.

CONCLUSION

The study shows that triploidy can be achieved in banana shrimp, *P. merguiensis* by cold shock treatment. Temperatures at 15°C for durations of 10, 15 and 20 minutes were identified as the optimum parameters to induce triploid in *P. merguiensis*.

ACKNOWLEDGEMENT

This research was fully funded by a grant from MOSTI (Science Fund) under the Project No. 05-01-12-SF1004. Authors wish to thank laboratory staff for their assistance during the use of Advance microscopes *Nikon Eclipse* 80i and Video Karyo Test software.

REFERENCES

Aloise, D. A. & Francisco, A. M. L. 2010. Ploidy Manipulation and Polyploid Detection in the White Shrimp, *Littopenaeus vannamei* (Boone, 1931) (Decapoda, Penaeidae). *Marine Biotechnology*, **13**: 41-47.

Aflalo, E. D., Hoang, T. T. T., Nguyen, V. H., Lam, Q., Nguyen, D. M., Trinch, Q. S., Raviv, S., & Sagi, A. 2006. A Novel Two-step Procedure for Mass Productions of the Giant Freshwater Prawn *Macrobrachium rosenbergii*. *Aqualculture*, **256**: 468-478.

Benzie, J. A. H. 1998. Peneid Genetic and Biotechnology. *Aquaculture*, **164**: 23-47.

Brake, J., Davidson, J., & Davis, J. 2004. Field Observations on Growth, Gametogenesis, and Sex Ratio of Triploid and Diploid *Mytilus edulis*. *Aquaculture*, **236**: 179–191.

Coman, F. E., Sellars, M. J., Norris, B. J., Coman, G. J., & Preston, N. P. 2008. The Effects of Triploidy on *Penaeus (Marsupenaeus) Japonicus* (Bate) Survival, Growth and Gender when Compared to Diploid Siblings. *Aquaculture*, **27**: 50–59.

Dumas, S., & Ramos, C. 1999. Triploidy Induction in the Pacific White Shrimp *Litopenaeus vannamei* (Boone). *Aquaculture Research*, **30**: 621-624.

Felip, A., Zanuy, S., Carrillo, M., & Piferrer, F. 2001. Induction of Triploidy and Gynogenesis in Teleost Fish with Emphasis on Marine Species. *Genetica*, **111**: 175-195.

Food and Agriculture Organization (FAO). 2011. Cultured Aquatic Species Information Programme *Penaeus vannemei* (Boone,1931). Fisheries and Aquaculture Deparment.

Choo, P. S. 1984. Pengeluaran Benih Udang Laut. Institut Penyelidikan Perikanan Gugor, Pulau Pinang.

Harrell, R. M., Van Heukelem, W., & Kerby, J. H. 1998. A Comparison of Triploid Induction Validation Techniques. *Progressive Fish- Culturist*, **60**: 221-226.

Li, F., Zhou, L., Xiang, J., Liu, X., & Zhu, J. 1999. Triploidy Induction with Heat Shocks to *Penaeus chinensis* and their Effects on Gonad Development. *Chinese Journal of Oceanology and Limnology*, **17**: 57–61.

Norris, B. J., Coman, F. E., Sellars, M. J., & Preston, N. P. 2005. Triploid Induction in *Penaeus japonicus* (Bate) with 6 Dimethyl Aminopurine. *Aquaculture Research*, **36**: 202–206.

Phongdara, M., Chotigeat, W., Chandumpai, A., Tanthana, C., & Duangtong, P. 1999. Identification of *Penaeus merguiensis* and *Penaeus indicus* by RAPD - PCR Derived DNA Markers. *Reasearch Asia*, 143-151.

Sellars, M. J., Li, F., Preston, N. P., & Xiang, J. 2010. Penaeid Shrimp Polyploidy: Global Status and Future Direction. *Aquaculture*, **310**: 1-7.

Sellars, M. J., Degnan, B. M., & Preston, N. P. 2006. Induction of Triploid Kuruma Shrimp, (*Marsupenaeus penaeus) Japonicus* (Bate) Nauplii through Inhibition of Polar Body I, or Polar Body I and II Extrusion using 6 Dimethylaminopurine. *Aquaculture*, **256**: 337–345.

Shelton, W. L. 2006. Regulated Sex Control in Commercially Important Fishes - A Physiological Perspective. *Israeli Journal of Aquaculture***, 58 (4)**: 351-365.

Tave, D. 1990. Chromosomal Manipulation. *Aquaculture Magazine*, **16**: 62-65.

Xiang, J. H., Zhou, L. H., Liu, R. Y., Zhu, J. Z., Li, F. H., & Liu, X. D. 1992. Induction of the Tetraploids of the Chinese Shrimp, *Penaeus chinensis*. In : Proceedings of the Asia Pacific Conference on Agricultural Biotechnology, Beijing 20-24 Aug. 1992. *China Science and Technology Press*, 841-846.

Xiang, J. H., Liu, R. Y., & Zhou, L. H. 1994. Chromosomes of Marine Shrimps with Special Reference to Different Techniques. *Aquaculture***, 111**: 321.

Xiang, J., Li, F., Zhang, X., Yu, K., Zhou, L., & Wu, C. 2006. Evaluation of Induced Triploid Shrimp *Penaeus* (*Fenneropenaeus) Chinensis* Cultured under Laboratory Conditions. *Aquaculture*, **259**: 108–115.

Zhang, X., Li, F., & Xiang, J. 2003. Chromosome Behavior of Heat Shock Induced Triploid in *Fenneropenaeus chinensis*. *Chinese Journal of Oceanography and Limnology*, **21 (31)**: 222-228.