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EFFECTS OF FOOD TYPE ON THE QUALITY OF SPERMATOPHORES AND SUBSEQUENT CRYOPRESERVATION AND FERTILIZATION IN BANANA SHRIMP, *Penaeus merguiensis* (De Man, 1888)

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Thesis Submitted in Fulfillment of the Requirement for the Degree of Doctor of Philosophy in the Institute of Tropical Aquaculture Universiti Malaysia Terengganu

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Dedication

"I humbly thank Allah Almighty,

The Merciful and the Beneficent,

Who gave me strength

Thoughts and co-operative people to enable me to achieve this goal.

As well as my family,

Who offered me unconditional love and support

Throughout the course, of this Thesis."

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EFFECTS OF FOOD TYPE ON THE QUALITY OF SPERMATOPHORES AND SUBSEQUENT CRYOPRESERVATIONAND FERTILIZATION IN BANANA SHRIMP, *Penaeus merguiensis* (De Man, 1888)

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The goal of this study was to optimize spermatophores cryopreservation protocol of banana shrimp, *Penaeus merguiensis*. Spermatophores cryopreservation is a key topic in the development of reproductive technologies which provide the possibility to protect genetics of rare or beneficial species (gene banking), selective breeding, domestication, and conservation of stocks, and loss minimization during disease outbreaks. In addition, it has been main focus of researchers to enhance the production of shrimp aquaculture and improve availability of seed stock during periods of high demand. The present study was undertaken with the following objectives; i) to determine the effects of different fresh natural diets on the quality of spermatophores, ii) to define the sperm viability prior to cryopreservation, iii) to determine optimum temperature and cryoprotactant concentration for cryopreservation protocol, and iv) to examine the morphology of cryopreserved sperms. Meanwhile, a total of 996 mature males with mean body weight (BW) of 23.9±4.6 g and mean total length (TL) of 15.1±0.5 cm and 78 mature female with mean BW of 28.1±6.1 g and mean TL of 14.2±0.6 cm were used in this study.

Determination effects of different fresh natural diets on the quality of spermatophores were carried out for six weeks with three treatments (fresh squid, polychaete and cockle). Spermatophore quality was evaluated by sperm viability and count, spermatophore weight and Specific Growth Rate, and proximate analysis were done on offered diets along with experimental shrimp. The sperm viability prior to cryopreservation was evaluated from live specimens (Group A); only spermatophores extracted (Group B) and homogenized spermatophore (Group C).

In the optimization of spermatophore cryopreservation protocol, the sterile calcium free saline (Ca-F saline) was used as an extender medium containing six tested cryoprotectants; dimethyl sulfoxide (DMSO), ethylene glycol (EG), methanol, glycerol, sucrose and magnesium chloride ($MgCl_2$) at concentrations of 5, 10, 15 and 20% each. The four cooling rates (20,16, 4, 2℃) and four cooling end points (-4,-20, -80, -100 to -150℃) were investigated with three exposure times of 5, 10 and 15 min, followed by storing in liquid nitrogen (LN) (-196℃). Five thawing temperatures (25, 27, 29, 31, 33℃) for 1, 2, 3, 4 and 5 min were selected to evaluate viability by using vital stains on 6, 12, 24 h, 7, 30, 60, 90, 120, 150 and 180 days during storage in LN (-196℃).

Morphological observations of cryopreserved sperms at 6, 12, 24 h and 7, 30, 60, 90, 120, 150 and 180 days were analysed by standard scanning electron microscope (SEM) technique with fresh sperms as control.

It was observed that squid treatment had the highest sperm viability (32.12%) and Sperm count (48.9%) (P <0.05) higher compared to the control. The concentration of

lipid in shrimp fed with squid was significantly higher by 5.74% as compared to the control (P <0.05). The sperm viability of fresh sperms was observed to be 97.3±2.7%, and at 7th h the viability was declining, which was 52.7±3.08.

The optimization of cryopreservation protocol of *P. merguiensis* spermatophores was attained after evaluation of cryoprotectants, equilibration period, cooling and thawing rates. It was apparent that $MgCl_2$ was the least toxic to sperm; it was subsequently used at a concentration of 15% in this study. The highest post-thaw sperm viability was recorded when spermatophores were cooled between 20 and -100 to -150℃ liquid nitrogen vapour (LNV) for 10 min followed by storing in liquid nitrogen for 24 h exhibit a mean viability of 84.3±2.90% in comparison to fresh spermatophores (93.8±2.5%)(P>0.05). Cryopreserved spermatophore held in LN for 90 days contain high sperm viability, although for longer periods, sperm viability declined. Highest sperm viability, 82.27±4.17 was observed after thawing at 27℃ for 2 min. There were no significant differences between fertilization and hatching rate for 90 days compared to fresh spermatophores (P >0.05). SEM observation of between 6 h and 90^{th} day cryopreserved sperms showed similar results as the fresh sperm that had no difference in mean length (μm) of sperm head, spike and body length (P >0.05).

From the results of the present study, it is concluded that fresh squid diet is highly preferable over other diets. It was observed that time had significant influence on the percentage of sperm viability and count. The cryopreserved spermatophore held in LN less than 90 days revealed high sperm viability. Moreover, the cryopreserved spermatophore kept for 90 days had similar rates of fertilization and hatching,

compared to fresh spermatophore. Meanwhile, the morphology of the cryopreserved sperm for 6 h – 90th day produced similar results to the fresh sperm. The method described in this study and potential application of this work on *P. merguiensis* represents a major advancement in studies involving optimization of cryopreservation protocol.

Abstrak tesis yang dikemukan kepada senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN JENIS MAKANAN ATAS KUALITI SPERMA DAN SETERUSNYA KRIOWETAN DAN PERSENYAWAAN DALAM UDANG PUTIH, *Penaeus merguiensis* (De Man, 1888)

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December 2012

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Capaian kajian ini adalah untuk mengoptimumkan protokol pengawetan spermatofor udang putih, *Penaeus merguiensis*. Pengawetan spermatofor adalah kunci utama dalam pembangunan teknologi pembiakan yang menyalurkan keupayaan untuk melindungi genetik spesis yang pupus dan bernilai (bank gen), pembiakan terpilih, untuk tujuan domestik, dan stok pemuliharaan, dan untuk mengurangkan kerugian ketika penyebaran wabak penyakit. Selain itu, telah menjadi fokus utama para pengkaji untuk meningkatkan penghasilan udang akuakultur dan menambah baik lagi stok benih bagi penghasilan ketika permintaan tinggi. Kajian dilakukan berdasarkan objektif berikut; i) mengenalpasti kesan makanan segar semulajadi terhadap kualiti spermatofor, ii) menentukan viabiliti sperma untuk tujuan pengawetan, iii) mengenalpasti suhu optimum dan kandungan bahan pengawet bagi protokol pengawetan, dan iv) mengkaji morfologi sperma awet. Sementara itu, sebanyak 996 jantan matang dengan min berat badan 23.9±4.6 g dan min jumlah panjang 15.1±0.5 cm dan 78 betina matang dengan min berat badan 28.1±6.1 g dan min jumlah panjang 14.2±0.6 cm digunakan dalam kajian ini.

Mengenalpasti kesan makanan segar semulajadi terhadap kualiti spermatofor telah dijalankan selama enam minggu menggunakan tiga rawatan (sotong segar, umpun-umpun, dan kerang). Kualiti spermatofor ditentukan dengan viabiliti sperma serta bilangan sperma, berat spermatofor dan Kadar Tumbesaran Spesifik, dan analisa dilakukan terhadap makanan bersama udang yang dikaji. Viabiliti sperma bagi tujuan pengawetan dinilai daripada spesimen hidup (Kumpulan A); hanya spermatofor (Kumpulan B), dan kandungan sperma dikeluarkan daripada spermatofor (Kumpulan C).

Bagi pengoptimuman protokol pengawetan spermatofor, kalsium bebas garam steril (Ca-F saline) yang telah digunakan sebagai media pengawetan mengandungi enam bahan pengawet yang telah diuji; dimethyl sulfoxide (DMSO), ethylene glycol (EG), methanol, glycerol, sucrose and magnesium chloride ($MgCl_2$) pada kepekatan 5, 10, 15 dan 20% setiap satu. Empat kadar penyejukan (20, 16, 4 2℃) dan empat penyejukan titik akhir ((-4,-20, -80, -100 to -150℃) dikaji dengan tiga pendedahan masa yang berbeza 5, 10, dan 15 min, diikuti dengan langkah penyimpanan di dalam cecair nitrogen (-196℃). Lima suhu pencairan (25, 27, 29, 31, 33℃) bagi 1, 2, 3, 4 dan 5 min dipilih untuk menentukan viabiliti dinilai dengan menggunakan (kesan penting) vital stains terhadap 6, 12, 24 h, 7, 30, 60, 90, 120, 150 dan 180 hari tempoh penyimpanan di dalam cecair nitrogen (-196℃).

Pemerhatian terhadap sperma awet pada 6, 12, 24 jam dan 7, 30, 60, 90, 120, 150 dan 180 hari dianalisa dengan menggunakan teknik standard mikroskop imbasan elektron dengan sperma segar sebagai kawalan.

Didapati bahawa rawatan menggunakan sotong mempunyai viabiliti sperma tertinggi (32.12%) dan bilangan sperma (48.9%) (P <0.05) lebih tinggi berbanding kawalan. Kandungan lemak di dalam udang yang diberi makan sotong adalah lebih tinggi 5.74% berbanding kawalan (P <0.05). Viabiliti sperma segar menunjukkan 97.3±2.7%, dan pada jam ke-7, viabiliti menurun iaitu 52.7±3.08.

Pengoptimuman protokol pengawetan spermatofor *P. merguiensis* telah dilakukan selepas kajian ke atas bahan pengawet, tempoh pengimbangan penyejukan dan kadar pencairan. Jelas menunjukkan bahawa $MgCl_2$ adalah kurang toksik kepada sperma; digunakan pada kandungan 15% dalam kajian ini. Viabiliti pencairan sperma yang paling tinggi direkodkan apabila spermatofor disejukkan diantara 20 dan -100 sehingga -150℃ denagn wap cecair nitrogen selama 10 min diikuti penyimpanan di dalam cecair nitrogen selama 24 jam menghasilkan min viabiliti 84.3±2.90% berbanding spermatofor segar (93.8±2.5%) (P>0.05). Spermatofor awet yang disimpan di dalam cecair nitrogen selama 90 hari mengandungi viabiliti sperma yang tinggi, walaupun pada tempoh yang lama, viabiliti sperma menurun. Viabiliti sperma tertinggi, 82.27±4.17 ditentukan selepas pencairan pada 27℃ selama 2 min. Tiada sebarang perbezaan diantara kadar persenyawaan dan kadar penetasan selama 90 hari berbanding spermatofor segar (P >0.05). Pemerhatian dibawah mikroskop imbasan elektron diantara 6 jam dan hari ke-90 pengawetan sperma menunjukkan keputusan yang sama seperti sperma segar dimana tiada perbezaan bagi min panjang (μm) kepala sperma, ekor, panjang badan (P >0.05).

Berdasarkan keputusan yang diperoleh daripada kajian ini, dapat disimpulkan bahawa makanan sotong segar lebih baik berbanding makanan lain. Pemerhatian

telah dijalankan ke atas peratusan viabiliti sperma dan juga bilangan sperma. Spermatofor yang diawet disimpan selama 90 hari di dalam cecair nitrogen menunjukkan viabiliti sperma yang tinggi. Tambahan lagi, spermatofor awet yang disimpan selama 90 hari mempunyai kadar persenyawaan dan kadar penetasan yang sama, jika dibandingkan dengan spermatofor segar. Sementara itu, morfologi sperma awet yang disimpan selama 6 jam hingga hari ke-90 menghasilkan keputusan yang sama dengan sperma segar. Kaedah yang dihuraikan di dalam kajian ini dan potensi kerja diaplikasi ke atas *P. merguiensis* menunjukkan potensi besar yang melibatkan protokol pengawetan.