EFFECTS OF DIFFERENT EQUILIBRATION DURATION, VAPOUR EXPOSURE TEMPERATURE AND VAPOUR EXPOSURE DURATION ON THE SPERM FREEZABILITY OF THAI MAHSEER (*Tor tambroides*)

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2011

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Name of Degree : **Master of Science (Msc)**

Title of Project Paper/Research Report/ Dissertation/ Thesis ("this Work"):

EFFECTS OF DIFFERENT EQUILIBRATION DURATION, VAPOUR EXPOSURE TEMPERATURE, VAPOUR EXPOSURE DURATION ON THE SPERM FREEZEABILITY OF THAI MAHSEER (*Tor tambroides*)

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ABSTRACT

The experiment was carried out to determine the effects of equilibration duration, vapour exposure temperature and vapour exposure duration in sperm cryopreservation protocol for *Tor tambroides* fish using modified Fish Ringer Extender (FRE); and subsequently used in experiment to develop optimal combination of equilibration duration, vapour exposure temperature and vapour exposure duration. Also, an experiment was performed to compare the frozen-thawed sperm characteristics between low temperature incubator and normal refrigerator during cryopreservation process; and the results have been utilised for subsequent final experiment in attempt of the transfer of technology from laboratory to the field condition for the sperm cryopreservation of *Tor tambroides* under field condition.

The freezing process under laboratory ambient was conducted in freezing laboratory, ISB Mini Farm (Livestock), University of Malaya in which a total of 20 Thai mahseer male fish was involved. The freezing process under field condition was carried out in Taman Negara Sg. Relau (Merapoh), Pahang which included a total of 17 Thai mahseer male fish. The mature Thai mahseer males were induced hormonally by injection of Ovaprim (0.5 ml/kg) 24 hours before the milt collection process. Semen was collected by gentle squeezing using centrifuge tube. The collected semen was diluted with Fish Ringer Solution (FRS) at 1:10 ratio after which was mixed with DMSO (10% of the diluent). The mixture was loaded into the 0.25/0.50 ml French straws and sealed. Subsequently the freezing process was carried out and stored in liquid nitrogen tank. Straws were thawed in water bath at 30ºC for 1 minute for evaluation of the sperm. Sperm motility characteristics for frozen-thawed sperm were evaluated using an Automated Semen Analyzer (IVOS, Hamilton-Thorne). The effect of factors and parameters were statistically analysed using one-way ANOVA and Duncan Multiple Range Test (DMRT).

Duration of 30 minutes equilibration gave higher total motility (67.31±1.27%) than 45 and 60 minutes (61.93±1.31% and 65.09±1.02%, respectively) durations for Thai mahseer sperm cryopreservation protocol. Among three temperatures used for vapour exposure phase in this study, -110°C was considered to be the most suitable temperature which obtained the highest values in total motility and progressive motility (68.53±1.13% and 15.75±0.61%, respectively) compared to -100°C (60.74±1.41% and 11.65±0.68%, respectively) and -120°C (65.07±0.99% and 12.56±0.56%, respectively). Meanwhile, 15 minutes of vapour exposure duration produced the highest values of total motility and progressive motility (65.25±1.25% and 14.83±0.71%, respectively) compared to 5 (63.87±1.31% and 12.31±0.58%, respectively) and 10 minutes durations (65.21±1.09% and 12.82±0.58%, respectively). Combination of 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 10 minutes of vapour exposure duration; combination of 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration; and combination of 30 minutes of equilibration duration, -120°C of vapour exposure temperature and 15 minutes of vapour exposure duration were considered as three optimal combinations in sperm cryopreservation of Thai mahseer and were used in subsequent experiment.

Combination of 30 minutes of equilibration duration, -110°C of vapour exposure temperature and 15 minutes of vapour exposure duration showed the highest values in total motility and progressive motility (72.80±2.85% and 21.20±4.02%, respectively) compared to other two combinations [(72.40±2.27% and 16.80±2.22%, respectively) and (70.80±2.35% and 18.20±2.48%, respectively)] used. Both low temperature incubator and normal refrigerator attained the same effect during the equilibration phase which showed no significant differences when using all equilibration durations (30, 45 and 60 minutes) in

total motility (ranging from 97.07±0.74% to 97.60±0.50%, from 87.80±1.74% to 88.40±1.59% and from 88.33±1.59% to 88.67±1.87%, respectively) and progressive motility (ranging from 34.60±4.04% to 38.60±4.16%, from 23.13±2.16% to 23.93±2.95% and from 21.13±1.83% to 21.47±2.48%, respectively) values. As for comparison, the freezing process under laboratory ambient produced higher values in total motility, progressive motility, rapid, medium, slow and ALH (75.03±1.49%, 19.47±1.46%, 24.80±1.83%, 10.23±0.74%, 38.53±1.52% and 3.26±0.12 μm, respectively) compared to freezing process under field condition (35.81±1.69%, 8.96±0.64%, 10.36±0.75%, 4.76±0.37%, 23.11±1.36% and 2.95±0.10 μm, respectively). However, the most interesting facts from this attempt, there were no significant differences in VAP, VSL, VCL, BCF, STR and LIN values between the freezing under laboratory ambient and field condition with a range from 41.54±0.91 μm/s to 43.53±1.33 μm/s, from 37.24±0.90 μm/s to 38.10±1.28 μm/s, 57.30±0.95 μm/s to 61.16±1.35 μm/s, from 24.96±0.62 Hz to 25.48±1.04 Hz, from 86.33±0.53% to 87.94±0.44% and from 62.03±1.20% to 62.93±0.69%, respectively.

In summary, the results demonstrate that the Thai Mahseer sperm could be cryopreserved using modified Fish Ringer Extender (mFRE) with good frozen-thawed sperm motility characteristics both under laboratory ambient as well as field condition.

ABSTRAK

Eksperimen ini dijalankan bertujuan untuk menentukan kesan tempoh pengimbangan, suhu pengewapan dan tempoh pengewapan dalam protokol penyejukbekuan sperma ikan kelah (*Tor tambroides*) menggunakan '*modified Fish Ringer Extender (mFRE)*'; dan seterusnya digunakan dalam eksperimen untuk membentuk kombinasi optimal untuk masa pengimbangan, suhu pengewapan dan jangka masa pengewapan. Di samping itu, eksperimen ini dilakukan untuk membandingkan ciri-ciri sperma yang dinyahbeku antara proses penyejukbekuan menggunakan inkubator suhu rendah dan peti sejuk biasa; dan keputusannya akan dimanfaatkan dalam ekperimen terakhir yang seterusnya iaitu percubaan untuk memindahkan teknologi penyejukbekuan sperm ikan kelah (*Tor tambroides*) dari persekitaran makmal ke keadaan persekitaran semula jadi.

Proses penyejukbekuan di bawah persekitaran makmal telah dijalankan di makmal penyejukbekuan, Ladang Mini ISB (Ternakan), Universiti Malaya di mana melibatkan 20 ekor ikan kelah jantan. Sejumlah 17 ekor ikan kelah digunakan bagi proses sejukbeku di bawah keadaan persekitaran semula jadi yang telah dilakukan di Taman Negara Sg. Relau (Merapoh), Pahang. Ikan kelah jantan yang matang distimulasi secara hormon dengan suntikan 'Ovaprim' (0.5 ml/kg) pada 24 jam sebelum proses pengumpulan semen dijalankan. Pengumpulan semen dilakukan dengan mengurut bahagian abdomen secara perlahan-lahan dan menggunakan tiub pengemparan. Semen yang telah dikutip akan dicairkan dengan '*modified Fish Ringer Solution (mFRS)*' pada kadar 1:10 dan campuran itu dicampurkan dengan DMSO (10% daripada campuran tersebut). Campuran akan dimasukkan ke dalam 0.25/0.50 ml '*French straws*' dan diterakan. Kemudian, straw-straw tersebut akan melalui proses penyejukbekuan dan disimpan di dalam tangki cecair nitrogen. Straw akan dinyahbeku di dalam pemanas air pada 30ºC selama 1 minit untuk

analisis sperma tersebut. Ciri-ciri motiliti sperma yang dinyahbeku akan dianalisis dengan menggunakan mesin penganalisis sperma secara automatik (IVOS, Hamilton-Thorne). Kesan faktor-faktor dan parameter-parameter yang diukur dianalisis secara statistik dengan menggunakan '*one-way ANOVA*' dan '*Duncan Multiple Range Test* (DMRT)'.

Tempoh pengewapan selama 30 minit memberikan motiliti menyeluruh (67.31±1.27%) yang lebih tinggi berbanding tempoh 45 dan 60 minit (61.93±1.31% dan 65.09±1.02%, masing-masing) dalam protokol penyejukbekuan sperma ikan kelah. Antara tiga suhu pengewapan yang digunakan dalam kajian ini, -110°C disimpulkan sebagai suhu yang paling sesuai di mana memperolehi nilai paling tinggi dalam motility menyeluruh dan motility progresif (68.53±1.13% dan 15.75±0.61%, masing-masing) berbanding -100°C (60.74±1.41% dan 11.65±0.68%, masing-masing) dan -120°C (65.07±0.99% dan 12.56±0.56%, masing-masing). Selain itu, tempoh pengewapan selama 15 minit menghasilkan nilai paling tinggi dalam motility menyeluruh dan motiliti progresif (65.25±1.25% dan 14.83±0.71%, masing-masing) berbanding tempoh 5 minit (63.87±1.31% dan 12.31±0.58%, masing-masing) dan 10 minit (65.21±1.09% dan 12.82±0.58%, masing-masing). Antara tiga kombinasi terbaik dalam proses sejukbeku ikan kelah ini ialah 30 minit tempoh pengimbangan, -110°C suhu pengewapan dan 10 minit tempoh pengewapan; 30 minit tempoh pengimbangan, -110°C suhu pengewapan dan 15 minit tempoh pengewapan; dan 30 minit tempoh pengimbangan, -120°C suhu pengewapan dan 15 minit tempoh pengewapan

Kombinasi 30 minit tempoh pengimbangan, -110°C suhu pengewapan dan 15 minit tempoh pengewapan telah menunjukkan nilai tertinggi dalam motility menyeluruh dan motiliti progresif (72.80±2.85% dan 21.20±4.02%, masing-masing) berbanding dua kombinasi lain yang telah digunakan [(72.40±2.27% dan 16.80±2.22%, masing-masing)

dan (70.80±2.35% dan 18.20±2.48%, masing-masing)]. Kedua-dua inkubator suhu rendah dan peti sejuk biasa mengalami kesan yang sama semasa fasa pengimbangan di mana tiada perbezaan yang signifikan menggunakan semua tempoh pengimbangan (30, 45 dan 60 minit) dalam nilai-nilai motiliti menyeluruh (berjulat dari 97.07±0.74% ke 97.60±0.50%, dari 87.80±1.74% ke 88.40±1.59% dan dari 88.33±1.59% ke 88.67±1.87%, masing-masing) dan motiliti progresif (berjulat dari 34.60±4.04% ke 38.60±4.16%, dari 23.13±2.16% ke 23.93±2.95% dan dari 21.13±1.83% ke 21.47±2.48%, masing-masing). Sebagai perbandingan, proses penyejukbekuan yang dijalankan di bawah persekitaran makmal menunjukkan perbezaan yang signifikan dalam motiliti menyeluruh, motiliti progresif, taburan sperma laju, taburan sperma sederhana, taburan sperma perlahan dan ALH (75.03±1.49%, 19.47±1.46%, 24.80±1.83%, 10.23±0.74%, 38.53±1.52% dan 3.26±0.12 µm, masing-masing) berbanding proses penyejukbekuan yang dilakukan di bawah keadaan persekitaraan semula jadi (35.81±1.69%, 8.96±0.64%, 10.36±0.75%, 4.76±0.37%, 23.11±1.36% dan 2.95±0.10 µm, masing-masing). Walaubagaimanapun, fakta-fakta menarik yang didapati daripada eksperimen ini, tiada perbezaan signifikan dalam nilai-nilai VAP, VSL, VCL, BCF, STR dan LIN antara penyejukbekuan di bawah persekitaran makmal dan keadaan persekitaran semula jadi berjulat dari 41.54±0.91 µm/s ke 43.53±1.33 µm/s, dari 37.24±0.90 µm/s ke 38.10±1.28 µm/s, dari 57.30±0.95 µm/s ke 61.16±1.35 µm/s, dari 24.96±0.62 Hz ke 25.48±1.04 Hz, dari 86.33±0.53% ke 87.94±0.44% dan dari 62.03±1.20% ke 62.93±0.69%, masing-masing.

Secara keseluruhan, keputusan-keputusan dari eksperimen ini menunjukkan sperma ikan kelah boleh disejukbekukan menggunakan ‘*modified Fish Ringer Extender* (mFRE)’ dengan menghasilkan ciri-ciri motiliti sperma yang dinyahbeku di bawah persekitaran makmal mahupun keadaan persekitaran semula jadi.

ACKNOWLEDGEMENTS

First of all, my utmost gratitude to Allah SWT, the Creator of this universe, with His willing and permission, I am able to complete this writing on my study. With great humility, I pledge upon improving myself as a humble servant, to adhere to His instruction and to stay away from unlawful routes.

I would like to convey my greatest gratitude and appreciation to both of my supervisor and co-supervisor; Prof Dr. Wan Khadijah Embong and Prof. Dr. Ramli Abdullah, for their endless efforts, continuous support, useful advices and guidance throughout this study. It is really an honour for me to be accepted as your student and laboratory member and thank you for sharing all valuable knowledge and experience in order to make this thesis possible.

My sincere gratitude to Dr. Zaaba Zainol Abidin from Department of Wildlife and National Parks Peninsular Malaysia (PERHILITAN), for his efforts, guidance and ideas in order to achieve the collaboration bond and success. Also, thank to staff of PERHILITAN in particular Ms. Nosrat and Mr. Jeffry, staff of Taman Negara Sg. Relau, Merapoh, Pahang Darul Makmur, in particular Mr. Basyir and Mr. Sharif. Thank you for all of your supports, ideas and efforts.

I would like to express my thousand thank to all ABEL members, in particular Mr. Nik Mohd. Azuadi, Mr. Mohd Nizam, Mr. Zhi Chao, Ms. Nor Azlina, Ms. Sow Chan, Ms. Asdiana, Ms. Phek Jin, Ms. Raja Ili Airina, Ms. Norfadilah, Ms. Norfarizah, Ms. Hui Hui, Ms. Wei Lun, Ms. Siew Ying, Ms. Siti Khadijah and other members for their positive comments, advices and efforts. Futhermore, thanks to ISB mini farm workers in particular Mr. Razali Jonit, Mr. Mohd Azman and ISB staff, especially Mr. Parani and Mr. Azhar for

their invaluable helping hands, moral and vocal supports throughout this study. Thank you for your invaluable helping hands.

I would like to reserve my gratitude to PPP Research Grant of University of Malaya (PS289/2008C) for funded this project, in order to make this project became reality, well-organised and beneficial.

Last but most importantly, I would like to share a mountain of appreciation to my parents, siblings and other family members for their unwavering supports, encouragement and belief. To all my friends, in ISB department or elsewhere, thank you for all the helping hands and all the concerns, for keeping me up when I was down, for being there.

LIST OF PUBLICATIONS

1. Shahrulzaman, S., W.E. Wan Khadijah and R.B. Abdullah. 2009. Effects of equilibration duration, vapour exposure duration and vapour exposure temperature on sperm cryopreservation of Thai mahseer (*Tor tambroides, kelah*), Proceedings of the 30th Malaysian Society of Animal Production, June 2-5, Kota Kinabalu, Malaysia. pp. 132-133 (Abstract).

2. Shahrulzaman, S., W.E. Wan Khadijah and R.B. Abdullah. 2010. Sperm cryopreservation of thai mahseer (*Tor tambroides, kelah*) under field condition. Proceedings of the 31th Malaysian Society of Animal Production, June 6-8, Kota Bahru, Malaysia. pp. 168-169 (Abstract).

3. Shahrulzaman, W.E. Wan Khadijah and R.B. Abdullah. 2010. Effects of equilibration duration on frozen-thawed sperm motility characteristics in Thai mahseer (*Tor tambroides, kelah*). Proceedings of the 7th Annual Asian Reproductive Biotechnology Society Conference, November 8-10, Kuala Lumpur, Malaysia. p. 93 (Abstract).

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ABREVIATIONS

ABEL	Animal Biotechnology-Embryo Laboratory
ALH	Amplitude of Lateral Head Displacement
ANOVA	Analysis of variance
BCF	Beat-Cross Frequency
BSA	Bovine Serum Albumin
BSMIS	Buffered Sperm Motility-Inhibiting Saline Solution
Cho/PL	Cholesterol/Phospholipid
CPA	Cryoprotectant Agent
DMA	Dimethylacetamide
DMRT	Duncan's Multiple Range Test
DMSO	Dimethyl-sulfoxide
HBSS	Hanks' Balanced Salt Solution
ISB	Institute of Biological Sciences
LIN	Linearity
LN_2	Liquid Nitrogen
mFRE	Modified Fish Ringer Extender
mFRS	Modified Fish Ringer Solution
$NaHCO_3$	Sodium Bicarbonate
PG	Propylene-glycol
SEM	Standard Error of the Means
SPSS	Statistical Package for Social Science
STR	Straightness
SVP	Seminal Vesicle Plasma

US	United States
VAP	Average Path Velocity
VCL	Curvilinear Velocity
VSL	Straight Line Velocity

Chapter 1
INTRODUCTION