



CAPTIVE BREEDING AND REARING OF *Tachypleus Gigas* FOR ITS BIOMEDICAL UTILIZATION AND CONSERVATION

BY

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy (Biotechnology)

Kulliyyah of Science
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SEP 2018

ABSTRACT

Horseshoe crab (HSC) has been exploited by several industries such as agriculture (fertilizer) and as livestock feed, yet, the bigger threats come from fisheries, biomedical industries and habitat destruction. Hence, this present study aimed at investigating various biological parameters, artificial breeding and rearing conditions to be used for horseshoe crab's conservation. The first stage of this study was aimed at studying the effect of captivity on hemolymph quality for the purpose of LAL production, and sperm traits of *T. gigas* for captive spawning and rearing studies. The second stage of this study focused on captive spawning and rearing of *T. gigas* for the restocking/repopulation purpose. The third stage aimed at identifying pathogens that caused mortality during captive breeding and rearing studies. Effect of captivity was assessed in wild and captive horseshoe crabs (5 month captivity) collected from Balok beach, Kuantan, Pahang. The blood was collected in 6 different anticoagulant formulations (A, B, C, D, E and F) and amoebocyte cells' density, viability and morphology were investigated. While the sperm was collected from wild and captive horseshoe crabs by massaging the gonads and sperm density and viability were studied. Optimum captive spawning and rearing of *T. gigas* conditions were then investigated. Importance and type of sand substrates (no substrate, coarse sand and fine sand) as well as the addition of fish oil (CLO) to *T. gigas*'s feed were studied to improve captive spawning. While differential microalgae feeds (Isochrysis, Spirulina and Isochrysis +Spirulina) were studied to induce molting and improve survival of *T. gigas* instars. Pathogens that infected *T. gigas*'s adults and eggs were isolated and cultured in various media and culture conditions. Genomic DNA extraction, PCR amplification and DNA sequencing were carried out. Molecular identification of the pathogens was carried out using partial sequencing of 16S rRNA gene for bacteria and ITS gene for fungi. Captivity was found to have profound negative effect on blood and sperm parameters. However, sperm traits showed that it is encouraging for restocking and sea ranching approach. *T. gigas* preferred spawning in moderately well sorted ($\sigma\phi = 0.52$) sand substrate with mean size ($X\phi$) of 2.57. Captive spawning was then further improved by 9 fold using fish oil (CLO). While Isochrysis at concentration of 7.5 million cells/L induced and accelerated molting in *T. gigas* instar stages. *Aeromonas caviae* strains were isolated from the gill fluid, while *Lysinibacillus fusiformis* strains were isolated from gill tissues of adult *T. gigas*. *Aspergillus aculeatus* strains were isolated from infected eggs. It can be concluded that LAL kits can't be developed and prepared by bleeding captive *T. gigas*. However, captive spawning and rearing could be a better approach and the most viable option for the restocking/repopulation of *T. gigas*. The diversity of the pathogens and their varying targets indicated the need for more studies on the design, feed and aquarium parameters used for maintaining horseshoe crabs in laboratory settings.

خلاصة البحث

يُستغل سرطان حدوة الحصان في العديد من الصناعات، مثل صناعة الأسمدة الزراعية، وعلف الماشية، ولكن التهديدات الكبرى لهذا الحيوان تأتي من تناوله كطعام، والصناعات الطبية الحيوية، وتدمير بيئته. ولذلك هدف هذا البحث إلى دراسة العوامل الحيوية المختلفة، والتربية الاصطناعية وظروف الإستكثار. هدفت المرحلة الأولى من البحث إلى دراسة تأثير التربية المغلقة على جودة الهيموليمف المستخدم لإنتاج سائل تفكك الأميبات السرطاني (LAL)، وخصائص الحيوانات المنوية لتاكيبليوس جيجاس (*T. gigas*) لدراسات الاستكثار والتربية المغلقة. ركزت المرحلة الثانية من هذه الدراسة على الاستكثار وتربية تاكيبليوس جيجاس لإعادة التخزين أو إعادة الإسكان في البرية. هدفت المرحلة الثالثة إلى تحديد الميكروبات التي كانت وراء نفوق السرطانات أثناء دراسات الاستكثار والتربية المغلقة. تم دراسة تأثير التربية المغلقة على سرطان حدوة الحصان البري والمربي (تربية مغلقة لمدة 5 أشهر) الذي تم جمعه من شاطئ بالوك في مدينة كوانتان بولاية باهانج. تم جمع الدم في 6 تركيبات مضادة للتلخثر (A، B، C، D، E، و F) وتم التحقيق في كثافة خلايا الأميبات، وحيويتها، وتشكلها. بينما تم جمع الحيوانات المنوية من سرطان حدوة الحصان البري والمربي داخليا عن طريق تدليك الغدد التناسلية، ومن ثم تمت دراسة كثافة وحيوية الحيوانات المنوية. ثم تمت دراسة أفضل عوامل التربية الاصطناعية وظروف الإستكثار لتاكيبليوس جيجاس. تمت دراسة أهمية السرير الرملي والنوع المناسب (بدون سرير رملي، سرير رملي خشن و سرير رملي ناعم) بالإضافة إلى أثر إضافة زيت السمك (CLO) إلى غذاء التاكيبليوس جيجاس في تحسين الاستكثار في التربية المغلقة. أيضا تم دراسة عدد من الطحالب الدقيقة كغذاء (الإيسوكريسيس، والسبيرولينا، و الإيسوكريسيس مع السبيرولينا) للحث على الانسلاخ وتحسين فرص العيش في الأطوار الأولية. ثم تم عزل الميكروبات التي أصابت حيوانات وبيض التاكيبليوس جيجاس وزراعتها في مختلف الوسائط الميكروبية وتحت مختلف الظروف. بينما تم تحدد عن طريق استخراج الحمض النووي الجينوم، تفاعل البوليميراز المتسلسل (PCR)، و من ثم معرفة التسلسل النووي (DNA Sequencing). تم تنفيذ التحديد الجزيئي للميكروبات باستخدام تسلسل جزئي لجين rRNA 16S للبكتيريا وجين ITS للفطريات. استنتج من خلال هذه الدراسة أن التربية المغلقة لها تأثير سلبي كبير على معايير الدم والحيوانات المنوية. ومع ذلك فقد أظهرت سمات الحيوانات المنوية أن التربية المغلقة كافية لإعادة التخزين والتربية البحرية. استنتج أيضا أن التاكيبليوس جيجاس يفضل الاستكثار في سرير رملي خشن معتدل الفرز ($\sigma\phi = 0.52$) ومتوسط الحجم $2.57(X\phi)$. ثم تم تحسين الاستكثار في التربية المغلقة بمقدار 9 أضعاف باستخدام زيت السمك (CLO). في حين أن طحلب الأيزوكريسيس (*Isochrysis*) بتركيز 7.5 مليون خلية لكل لتر حفز وسرع الانسلاخ في الأطوار الأولية. تم عزل سلالات *Aeromonas caviae* من السائل الخيشومي، في حين تم عزل سلالات *Lysinibacillus fusiformis* من أنسجة خياشيم التاكيبليوس جيجاس. بينما إتضح أن بيوض التاكيبليوس جيجاس المنتجة في الأحواض أصيبت بفطر ال *Aspergillus aculeatus*. يمكن تلخيص نتائج هذه الدراسة في أن التربية المغلقة لها تأثير سلبي كبير على جودة هيموليمف التاكيبليوس جيجاس وبالتالي ليس من الممكن تطوير وإنتاج ال LAL بعملية إنزاف هيموليمف التاكيبليوس جيجاس المربي داخليا، ويعد الخيار الأكثر قابلية للتطبيق هو التبييض بالاستكثار المغلق لإعادة تخزين أو إعادة إسكان التاكيبليوس جيجاس في بيئته. دعى تنوع، ومعدل والأهداف المختلفة للعدوى الميكروبية إلى الحاجة إلى مزيد من الدراسات على تصميم ومعايير الأحواض و نوعية الأغذية المستعملة في العناية بسرطان حدوة الحصان مخبريا.

APPROVAL PAGE

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated to my wife, Anis Fadhlina Izyani

ACKNOWLEDGEMENTS

First of all, praise to the almighty Allah subhanahu wa taala for giving me the strength, blessings and guidance to complete this work.

My name might be the only one on the cover of this thesis, but there are many people behind this success, and ahead of everybody are my parents. Without the love and blessing of my parents it wouldn't be possible.

Special thanks go to the person who guided me all the way, my dear supervisor Prof. Dr. Kamaruzzaman Yunus. Thanks a lot for all the knowledge and support. My gratitude also extends to my co-supervisors Asst. Prof. Dr. Akbar John, Assoc. Prof. Dr. Zaleha Kassim and Assoc. Prof. Dr. Solachuddin J.A. Ichwan for their valuable suggestions, feedbacks and constructive comments.

I would also like to thank Kulliyah of Science lecturers, Dr. Mustafizur rahman, Dr. Hamzah, Dr. Azzmer, Dr. Latif and the rest who always been a reference for me to ask for suggestions and recommendation when doing my lab work. My gratitude also goes to my friends and futsal teammates who were there for me when i wanted to release my tension whether via football or other recreational activities. Big thanks also goes to my lab mate Razali and the rest.

I would also like to thank Kulliyah of Science's lab staff: Ahmad Muzzamil and Mueizzah who were in charge of the labs that i used and were very helpful and flexible with me in using the facilities in their laboratories. Also thanks to the head of INOCEM Research Station (IRS) Prof. Jalal Ahmed Khan and his staff, Khairul Anwar, Faizal, Hilmi and the rest for giving me assistance and access to various facilities there as well.

I would also like to thank Kulliyah of Science's administrative staff, Norsa'adah, Taufik and Azim for their guidance, updates and assistance regarding postgraduate matters and to the rest of the staff who always showed me a smile and a helping hand when needed.

Finally, my highest gratitude also goes to Malaysian International Scholarship (MIS) and the Ministry of Higher Education Malaysia (MOHE) for giving me a fully funded scholarship. Thank you for the opportunity and trust in my PhD candidature.

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LIST OF ABBREVIATIONS

+G	Discrete Gamma Distribution	LAL	<i>Limulus</i> Amoebocyte Lysate
μ	Micron	LC	Least Concern
μm	Micrometer	LPS	Lipopolysaccharide
AA	Arachidonic Acid	m	Meter
AGT	Adult Gill Tissue	M	Molar
AS	Artificial seawater	MA	Marine Agar
ASMFC	Atlantic States Marine Fisheries Commission	Mar	March
Aug	August	MB	Marine Broth
BIC	Bayesian Information Criterion	MEGA	Molecular Evolutionary Genetics Analysis
BLAST	Basic Local Alignment Search Tool	mg	Milligram
C	Celsius	MHA	Mueller Hinton Agar
C	Contracted	MHB	Mueller Hinton Broth
Cd	Cadmium	mL	Milliliters
CFU/mL	Colony-Forming Units Per Millilitre	ML	Maximum Likelihood
CLO	Cod Liver Oil	mm	Millimeter
cm	Centimeter	mM	Millimolar
CR	Critically Endangered	MSL	mean sea level
Cr	Chromium	NA	Nutrient Agar
CR	<i>Carcinoscorpius rotundicauda</i>	Na ₂ EDTA	Disodium Ethylenediaminetetraacetic Acid
Cu	Copper	NaCl	Sodium Chloride
CW	Carapace Width	NB	Nutrient Broth
DD	Data Deficient	NE	Northeast
DF	Degranulated Flattened	NE	Not Evaluated
DHA	Docosahexaenoic Acid	Nov	November
DMSO	Dimethyl sulfoxide	NT	Near Threatened
DNA	Deoxyribonucleic Acid	OPL	Opisthosomal Length
DO	Dissolved oxygen	Pb	Lead
EDTA	Ethylenediaminetetraacetic	PDA	Potato Dextrose Agar

	acid		
ELISA	Enzyme-Linked Immunosorbent Assay	PDB	Potato Dextrose Broth
EN	Endangered	ppt	Part Per Thousand
EPA	Eicosapentaenoic Acid	proPO	Pro-phenoloxidase
ERDG	Ecological Research & Development Group	PVF	Perivitelline Fluid
EU	Endotoxin Unit	PW	Prosomal Width
EW	Extinct in the Wild	rpm	Revolutions Per Minute
EX	Extinct	rRNA	Ribosomal Ribonucleic Acid
FS	Filtered Seawater	SD	Standard Deviation
GD	Genetic Distance	Se	Selenium
GF	Granulated Flattened	SEM	Scanning Electron Microscope
GillF	Gill Fluid	-SH	Sulfhydryl Group
GR	Growth Rate	Sp.	Species (one)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	spp.	Species (multiple)
hr	Hour	SR	Survival Rate
HSC	Horseshoe crab	SSC	Species Survival Commission
HUFA	Highly-Unsaturated Fatty Acids	SW	Southwest
IM	Inter-monsoon	SW	Seawater
INOCEM	Institute of Oceanography and Maritime Studies	TailL	Tail Length
IO-Car	Intraocular Width to Carapace Width Ratio	TAL	<i>Tachypleus</i> Amoebocyte Lysate
IOW	Inter-Ocular Width	TG	<i>Tachypleus gigas</i>
IRS	INOCEM Research Station	TL	Total Length
ITS	Internal Transcribed Spacer	TSA	Tryptic Soy Agar
IUCN	International Union for Conservation of Nature	USA	United State of America
JGT	Juvenile Gill Tissue	USP	United States Pharmacopeia
Jun	June	VU	Vulnerable
K2P	Kimura 2-Parameter	WB	Whitish Biofilm
L	Liters	Zn	Zinc

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Fossil specimens of Horseshoe crab (HSC) that are as old as 540 million years have been found, hence HSC is known as “living fossils” (Rudkin & Young, 2009). This iconic species survived numerous extinction periods, ice ages, fluctuating atmospheric CO₂ and adjusted to dangerously low sea level (Loveland & Botton, 2015). Despite that, coexisting with human might be the biggest challenge to HSC species survival. There are four HSC crab species, namely, *Limulus polyphemus*, *Tachypleus tridentatus*, *Tachypleus gigas* and *Carcinoscorpius rotundicauda*. The International Union for Conservation of Nature (IUCN) red list for threatened species lists *Limulus polyphemus* as “Vulnerable (VU)”, while the other three species are all categorized as “Data Deficient (DD)” (Smith, Beekey, Brockmann, King, Millard, & Zaldívar-Rae, 2016; World Conservation Monitoring Centre, 1996a,b; Williams, 2013).

Horseshoe crab has been heavily harvested in fishing industry both as a bait and commodity, fertilizer for agriculture and as livestock feed (Carmichael & Brush, 2012; Kreamer & Michels, 2009). Yet, the biggest threat to HSC populations is possibly habitat degradation due to anthropogenic activities (Carmichael & Brush, 2012; Nelson et al., 2016b) and biomedical bleeding for the production of *Limulus/Tachypleus* Amoebocyte Lysate (LAL/TAL) (Akbar John, Jalal, Yunus, & Kasim, 2010; Kreamer & Michels, 2009).

This lead to serious decline in HSC populations, and a sustainable model for HSC utilization must be implemented urgently. One approach that can aid in the conservation of horseshoe crab is via aquaculture of horseshoe crab and captive

breeding, where each biomedical company can have a predetermined horseshoe crabs for LAL industry. This approach not only protect wild horseshoe crab populations, but also eliminate quantitative as well as qualitative variation between various manufacturers and batches previously reported, this is because the environmental conditions and feed of the animals are identical (Hurton, Berkson, & Smith, 2005; Novitsky, 2009). However, horseshoe crabs kept in captivity suffer from numerous infectious diseases caused directly or indirectly by various protozoa, bacteria, fungus, algae and parasites. Captive crabs are also in the risk of suffering from non-infectious disease such nutritional deficiencies, physical trauma, abnormal developmental, and a number of liver, kidney and intestinal diseases (Nolan & Smith, 2009). Development of these diseases was monitored in this study and counter measures were attempted.

Another approach is to study the potential of captive breeding and rearing techniques for the purpose of restocking/repopulation (Li, 2008; Nelson et al., 2016b; Shuster & Sekiguchi, 2003). Captive rearing of horseshoe crabs has been reviewed by Carmichael and Brush (2012) and the main issues reported were variation and inconsistency of data collection and reporting. For example, the aquarium conditions that are frequently reported are temperature, salinity and diet composition, while the author suggested the addition of other parameters such as dissolved oxygen, substrate as well as the quantity and frequency of feed. The authors also stressed on the importance of publishing these data in peer-reviewed journals to facilitate data sharing. These suggestions were considered and implemented in this study. The study was designed to explore both approaches and address challenges and limitations encountered by previous studies and the findings from this study could help directly in the conservation of horseshoe crab.

1.2 SIGNIFICANCE OF STUDY

Medically, horseshoe crab is virtually the only source of *Limulus* Amoebocyte Lysate (LAL), which is very crucial for the health and wellbeing of human. LAL is used in various industries such as pharmaceuticals, parenterals, vaccines, food quality, and many more to detect endotoxins. The LAL could also be used to monitor water quality, air quality and environmental quality assessment (Akbar John, Yunus, Jalal, & kassim, 2012; Swan, 2001; Wachtel & Tsuji, 1977).

Environmentally, horseshoe crabs are part of a bigger ecosystem and its population decline is associated with the decline of other species such as migratory shorebirds. Birds such as red knot (*Calidris canutus*), herring gulls (*Larus argentatus*), crows (*Corvus splendens*) and great black-backed gulls (*Larus marinus*), fish such as killifish (*Fundulus heteroclitus*), Atlantic silversides (*Menidia menidia*), loggerhead turtles (*Caretta caretta*) and Hermit crabs (*Pagurus longicarpus*) all feed on horseshoe crab eggs and trilobite larvae, hence could be affected by any decline in horseshoe crab populations. *L. polyphemus*, *T. tridentatus* and *C. rotundicauda* were highly associated with the population of invertebrates such as molluscs, polychaetes, bivalves and gastropods, while the number of juveniles is closely related to the population of phytoplanktons such as algae (Botton, 2009; Mizrahi & Peters, 2009).

In research, horseshoe crab, being a living fossil, could be a key to advancements in various fields such as biology and physiology. It has been in diagnostic medicine to detect fungi (McCarthy, Petraitiene, & Walsh, 2017; Ramanan, Wengenack, & Theel, 2017), isolating new antimicrobial agent (Józefiak & Engberg, 2017), immunology studies (Armstrong, 2016), in evolution studies (Pathak, Singh, Thirumalai, Armstrong, & Agrawal, 2016) and cancer research (Eitel, Hendrickson, Heyl-Clegg, Evans, & Guthrie, 2017).

Hence, its utmost important to carry on any research that can aid directly or indirectly in conserving horseshoe crabs.

1.3 RESEARCH OBJECTIVES

The study aimed to achieve the following objectives:

- 1- To evaluate the effect of captive breeding on hemolymph quality and sperm traits of *T. gigas*.
- 2- To determine and improve spawning success of *T. gigas* in captive conditions.
- 3- To study the effect of various microalgal feed on growth and mortality of *T. gigas* instars.
- 4- To study pathogenesis in *T. gigas* during captive breeding and rearing.

1.4 RESEARCH QUESTIONS

1. To what extent does captivity impair the quality of *T. gigas* hemolymph quality and sperm traits?
2. What are the necessary husbandry systems for captive spawning of *T. gigas*?
3. What hatchery system and feed can support captive rearing of juvenile *T. gigas*?
4. What are the pathogens that infects *T. gigas* adult, juvenile and eggs kept in captivity?

1.5 RESEARCH HYPOTHESES

Hypothesis 1: Captivity does not significantly affect blood and sperm traits in *T. gigas*.

Hypothesis 2: The use of sand substrate may significantly improve spawning of *T. gigas*, especially egg production.

Hypothesis 3: The addition of fish oil to *T. gigas* feed may significantly improve spawning, especially egg production.

Hypothesis 4: Addition of microalgae as feed may significantly improve molting and survival in *T. gigas* instars.

CHAPTER TWO

LITERATURE REVIEW

2.1 HSC BACKGROUND

2.1.1 Taxonomy

Horseshoe crab is a marine animal that descends from Arthropoda's phylum, classified under the class of Merostomata and order of Xiphosurida. They belong to the Limulidae family (Hemstock, 2014; Hong et al., 2009). Horseshoe crabs are more closely related to spiders and insects than to crabs (Yunus, Akbar John, Kasim, & Jalal, 2011). There are four species of horseshoe crab (Figure 2.1) that are classified under three different genera and assigned to two families. There is the *Limulus polyphemus* that belongs to Limulidae family, and *Tachypleus tridentatus*, *T. gigas*, *Carcinoscorpius rotundicauda* under Tachypleinae family (Sekiguchi & Shuster, 2009).

In Malaysia, three out of the four species of horseshoe crabs are found along the Malaysian coastal waters. *T. tridentatus* is found only in Sabah and Sarawak regions, while *T. gigas* and *C. rotundicauda* are distributed along the coast of Peninsular Malaysia (Kasim, Izyan, Siti Hamidah, Yunus, & Ahmed, 2011; Kassim, Shahuddin, Shaharom, & Chatterji, 2008; Zaleha, Hazwani, Hamidah, Kamaruzzaman, & Jalal, 2011).

2.1.2 Horseshoe Crab's Body Morphology

The body of horseshoe crab consists of three parts, the front part is Prosoma, a middle part called Opisthosoma and Telson (tail-like) (Figure 2.2). The horseshoe crab