UNIVERSITI PUTRA MALAYSIA

EARLY DEVELOPMENT AND FEEDING ABILITY OF YELLOW SEAHORSE, Hippocampus kuda (BLEEKER 1852), USING SELECTED LIVE FOODS DURING CRITICAL LIFE PHASE

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Faculty: Agriculture

Low survival remains a significant hurdle in the nursery culture of yellow seahorse *Hippocampus kuda*. To potentially improve the survival and growth of *H. kuda* culture, their early feeding ability which deduced from the development of external and internal morphology, ontogeny and changes of digestive enzyme activities, as well as the effects of selected live foods, specifically during the critical phase of first 10 days were evaluated.

The external morphology of newborn *H. kuda* was examined by scanning electron microscope (SEM) while their morphological development from 0 to 9 day-after-birth (DAB), both externally and internally, was inspected using the light microscope and analysed via histological approach. Despite the presence of some premature newborns with yolk sac attached to their bodies, normal *H. kuda* newborns had no external yolk sac and were able to swim freely and feed independently. The mouth gape size and mouth area increased from 0.26 to 0.53 mm and 0.070 to 0.165 mm^2, respectively. The head length to snout length (H:S) ratio remained similar at 2.2 throughout the growth of *H. kuda* juveniles while the tail length to head length (T:H) ratio increased from 1.3 to 2.0 as the juvenile developed. The digestive system of *H. kuda* juveniles consisted of a snout, straight intestinal tube with an opened anus, as well as a gallbladder, liver and pancreas. No stomach was observed. Internal yolk globules were found in newborn *H. kuda* but were depleted by 1 DAB. Although the juvenile height and intestine length increased with the age, the relative intestine length remained similar at 0.30.

Ontogeny development and activity of trypsin and chymotrypsin in *H. kuda* juveniles during the first 10 days and changes of digestive enzyme activities before and after feeding were evaluated using biochemical assays. Both the specific and total trypsin activities showed a similar trend where the activities were higher on 0–3 DAB, lower on 5 DAB and then slightly increased from 7 to 9 DAB. Meanwhile, the specific and

total chymotrypsin activities were low on 0 DAB, increased on 1–3 DAB, before decreasing and remaining low from 5 to 9 DAB. The trypsin to chymotrypsin (T:C) ratio was highest on 0 DAB but dropped drastically on the following day and remained low until the end of experiment. However, the digestive enzyme activities became slightly higher after feeding on live food organisms.

On the other hand, effects of different feeding regimes on *H. kuda* juveniles were investigated via two separate feeding trials. In Experiment 1, three feeding regimes (i) rotifers (20 ind mL^{-1}), (ii) *Artemia* nauplii (4 ind mL^{-1}), and (iii) a mixture of rotifers (15 ind mL^{-1}) and *Artemia* nauplii (1 ind mL^{-1}) were tested on *H. kuda* juveniles from 0 to 9 DAB. Prey consumption and heights of seahorses were measured every second day, while the survival and specific growth rate (SGR) were calculated after 9 DAB. A total mortality was observed among seahorses on 3 DAB and 5 DAB when only rotifers or *Artemia* were provided, respectively. The survival of seahorses fed the mixed live foods was 60% with a SGR of 6.3% d^{-1}. Larger *Artemia* were selected over smaller rotifers as *H. kuda* juveniles developed. Strong positive correlations were found between the consumption of larger *Artemia* and the mouth gape size ($r = 0.8551$, $P<0.05$), and mouth area ($r = 0.8509$, $P<0.05$), but the consumption of smaller rotifers was negatively correlated to either the mouth gape size ($r = -0.8345$, $P<0.05$) or mouth area ($r = -0.8194$, $P<0.05$) of *H. kuda* juveniles from 3 to 9 DAB. In Experiment 2, newborn seahorses were initially fed a mixture of rotifers (15 ind mL^{-1}) and *Artemia* nauplii (1 ind mL^{-1}), followed by either additional (i) fish oil emulsion enriched *Artemia* metanauplii (0.5 ind mL^{-1}) or (ii) copepods *Oithona simplex* (0.5 ind mL^{-1}) from 3 DAB onwards. The introduction of copepods significantly ($P<0.05$) improved the SGR of *H. kuda* to 8.1 % d^{-1} compared to 6.8 % d^{-1} without copepod, although the survival was similar.

Overall, the results indicated some progressive morphological changes along with a transition from partial lecithotrophy to full exogenous feeding in *H. kuda* juveniles during the first 10 days. The limited digestive capacity of *H. kuda* juveniles could have compensated by the autolysis of live food organisms. A feeding regime consisted of rotifers and *Artemia* nauplii mixture was suitable for *H. kuda* juveniles in terms of survival during this critical phase. To enhance growth, *O. simplex* should be used. These findings provided important knowledge to further improve the culture technique and perhaps development of an artificial feed for *H. kuda* species.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PERKEMBANGAN AWAL DAN KEUPAYAAN MAKAN KUDA LAUT, *Hippocampus kuda* (BLEEKER 1852), DENGAN MENGGUNAKAN MAKANAN HIDUP TERPILIH PADA FASA KEHIDUPAN KRITIKAL

Oleh

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Kadar kemandirian juvenil yang rendah merupakan suatu cabaran yang ketara dalam peringkat pembesaran kuda laut *Hippocampus kuda*. Untuk mempertingkatkan kadar kemandirian dan pertumbuhan awal juvenil *H. kuda*, keupayaan pemakanan awal dari segi perkembangan morfologi luaran dan dalaman, ontogeni dan perubahan aktiviti enzim pencernaan, serta kesan pemakanan makanan hidup terpilih, khususnya dalam tempoh kritikal 10 hari yang pertama telah dikaji.

Morfologi luaran *H. kuda* yang baru dilahir telah dinilai dengan menggunakan mikroskop elektron imbasan (SEM) manakala perkembangan morfologi juvenile dari 0 hingga 9 hari selepas lahir (DAB), luaran dan dalaman, telah diperhatikan dengan mikroskop cahaya atau dianalisis melalui pendekatan histologi. Walaupun terdapat beberapa kuda laut baru lahir pramatang dengan kantung yolka terletak di badan, *H. kuda* baru lahir yang normal tidak mempunyai kantung yolka luaran dan boleh berenang dan makan secara bebas. Saiz pembukaan mulut dan luas permukaan mulut masing-masing meningkat dari 0.26 ke 0.53 mm dan 0.070 ke 0.165 mm^2. Nisbah panjang kepala terhadap panjang muncung mulut (H:S) kekal pada 2.2 sepanjang pertumbuhan juvenil *H. kuda*, manakala nisbah panjang ekor terhadap panjang kepala (T:H) meningkat dari 1.3 ke 2.0, dengan perkembangan juvenil. Sistem pencernaan juvenil *H. kuda* terdiri daripada muncung, tiub usus yang lurus dan bersambungan dengan dubur yang terbuka, serta hempedu, hati dan pankreas. Perut tidak kelihatan. Globul yolka dalam badan kuda laut yang baru lahir didapati kempis pada 1 DAB. Walaupun ketinggian dan panjang usus meningkat sejajar dengan usia juvenil *H. kuda*, panjang usus relatif adalah berkekalan pada 0.30.

Perkembangan dan aktiviti ontogeni tripsin dan kimotripsin juvenil *H. kuda* dalam tempoh 10 hari pertama serta perubahan aktiviti enzim pencernaan sebelum dan selepas makan telah dinilaikan dengan kaedah biokimia. Kedua-dua aktiviti spesifik and keseluruhan tripsin menunjukkan trend yang serupa di mana aktiviti enzim

adalah lebih tinggi pada 0–3 DAB, rendah pada 5 DAB, kemudian meningkat sedikit pada 7–9 DAB. Sementara itu, aktiviti spesifik dan keseluruhan enzim kimotripsin adalah rendah pada 0 DAB, meningkat pada 1–3 DAB, kemudian turun semula dan kekal di paras rendah dari 5 hingga 9 DAB. Nisbah enzim tripsin terhadap kimotripsin (T:C) adalah paling tinggi pada 0 DAB, tetapi menurun secara mendadak pada hari yang berikut dan kekal di paras rendah sehingga akhir eksperimen. Namun, aktiviti enzim pencernaan juvenil *H. kuda* didapati meningkat sedikit selepas diberi makanan hidup.

Sementara itu, kesan regim pemakanan yang berlainan ke atas juvenil *H. kuda* telah dikaji melalui dua eksperimen yang berasingan. Dalam Eksperimen 1, tiga regim pemakanan (i) hanya rotifer (20 ind mL^{-1}), (ii) hanya naupli *Artemia* (4 ind mL^{-1}), dan (iii) campuran rotifer (15 ind mL^{-1}) dan naupli *Artemia* (1 ind mL^{-1}) telah disedia untuk juvenil *H. kuda* dari 0–9 DAB. Kadar pemakanan mangsa dan ketinggian kuda laut diukur dua hari sekali, manakala kadar kemandirian dan kadar pertumbuhan spesifik (SGR) dikira selepas 9 DAB. Kesemua juvenil mati pada 3 DAB dan 5 DAB apabila masing-masing hanya diberi rotifer atau *Artemia*. Kadar kemandirian kuda laut yang diberi campuran rotifer dan *Artemia* adalah 60% dengan SGR 6.3% d^{-1}. Makanan yang bersaiz lebih besar *Artemia* adalah lebih dipilih berbanding dengan rotifer yang bersaiz kecil apabila juvenil *H. kuda* semakin membesar. Korelasi positif yang kuat telah dikesan di antara kadar permakanan *Artemia* yang bersaiz lebih besar dan saiz pembukaan mulut ($r = 0.8551$, $P<0.05$) atau luas permukaan mulut ($r = 0.8509$, $P<0.05$), tetapi kadar permakanan rotifers yang bersaiz lebih kecil mempunyai kolerasi negatif dengan sama ada saiz pembukaan mulut ($r = -0.8345$, $P<0.05$) dan luas permukaan mulut ($r = -0.8194$, $P<0.05$) juvenile *H. kuda* dari 3 hingga 9 DAB. Dalam Eksperimen 2, juvenil *H. kuda* yang baru lahir diberi regim makanan campuran rotifers (15 ind mL^{-1}) dan *Artemia* nauplii (1 ind mL^{-1}), diikuti dengan penambahan sama ada (i) metanaupli *Artemia* yang telah diperkayakan dengan emulsi minyak ikan (0.5 ind mL^{-1}) atau (ii) kopepod *Oithona simplex* (0.5 ind mL^{-1}) mulai 3 DAB. Penambahan kopepod dalam regim pemakanan juvenil *H. kuda* telah mencatatkan peningkatan SGR yang signifikan ($P<0.05$) iaitu 8.1% d^{-1} berbanding dengan 6.8% d^{-1} dalam rawatan yang tidak diberi kopepod, walaupun kadar kemandirian kedua-dua rawatan adalah serupa.

Secara keseluruhan, keputusan telah menunjukan beberapa perubahan morfologi yang progresif setimpal dengan peralihan cara pemakanan dari separa lesitotrofi ke eksogenus yang sepenuh dalam sistem pemakanan juvenil *H. kuda* semasa 10 hari pertama. Keupayaan pencernaan juvenil *H. kuda* yang terhad berkemungkinan besar dibantu oleh autolisis organisma makanan hidup. Regim pemakanan yang terdiri daripada kombinasi rotifer dan *Artemia* adalah sesuai untuk juvenil *H. kuda* dalam fasa tumbesaran kritikal ini. Kopepod *O. simplex* perlu ditambah untuk mempertingkatkan tumbesaran kuda laut. Hasil penyelidikan ini memberi sumbangan pengetahuan yang penting untuk mempertingkat lagi teknik kultur dan berkemungkinan membantu dalam pembangunan makanan buatan untuk species *H. kuda*.

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I certify that a Thesis Examination Committee has met on (20th December 2017) to conduct the final examination of Teh Jun Chin on her thesis entitled "Early Development and Feeding Ability of Yellow Seahorse, *Hippocampus Kuda* (Bleeker 1852), Using Selected Live Foods During Critical Life Phase" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the candidate be awarded the relevant degree.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
AWI	Animal Welfare Institute
CITES	Convention on International Trade in Endangered Species
COMAS	Centre of Marine Science
DAB	day-after-birth
DHA	docosahexaenoic acid
dph	day post-hatched
EPA	eicosapentaenoic acid
FAME	fatty acid methyl esters
FID	Flame ionization detector
H&E	haematoxylin and eosin
H:S	head length to snout length ratio
HUFAs	highly unsaturated fatty acids
IACUC	Institutional Animal Care and Use Committee
IL:H	intestine length to height ratio
ind	individual
IUCN	International Union for Conservation of Nature
OR	olfactory receptor
PAS	periodic acid-Schiff
PNR	point-of-no-return
PUFAs	poly-unsaturated fatty acids
SD	standard deviation
SEM	scanning electron microscopy
SGR	specific growth rate
T:C	trypsin to chymotrypsin ratio
TCM	traditional Chinese medicine
T:H	tail length to head length ratio
UNEP-WCWC	United Nations Environment Programme – World Conservation Monitoring Centre
UPM	Universiti Putra Malaysia
UV	ultra-violet

CHAPTER 1

INTRODUCTION

1.1 Research background

Hippocampus kuda is one of the most heavily traded and over-harvested seahorse species in Southeast Asia (Choo & Liew 2003; Perry *et al*. 2010; Lawson *et al*. 2015). Live seahorses are usually traded as ornamental fish, but are also harvested and dried for traditional Chinese medicines (TCM) and tonic foods (Cohen 2012; Rosa *et al*. 2013). More than 32 million seahorses are traded by nearly 70 different countries worldwide during the years 2004-2011 and over 96% of these seahorses are captured in the wild (AWI 2013). In addition to the threats of trawler by-catches and over harvesting, the natural habitats for these exotic marine creatures that include seagrasses, mangroves, reefs and estuaries, are slowly being degraded due to extensive human activities, pollution and climate change (Giles *et al*. 2006; Koldewey & Martin-Smith 2010; Cohen 2012; Ngui 2014). Indeed, a decreasing availability of seahorses has been observed in Malaysia since the last two decades (Perry *et al*. 2010; IUCN 2014).

Seahorse breeding and aquaculture can potentially offer an alternative solution to support the high market demand, which may help to reduce the harvest of wild animals and perhaps restore the fish populations (Koldewey & Martin-Smith 2010). Although successful culture of seahorses has been reported in several studies, high mortalities during the first 10 days after birth remains a significant bottleneck in the mass culture of yellow seahorse *H. kuda* (Lin *et al*. 2006; Garcia & Hilomen-Garcia 2009; Celino *et al*. 2012). Despite intrinsic quality of the juvenile seahorses, poor nutrition which can be due to insufficient food, inability to capture food, and/or poor digestion of unsuitable food (Olivotto *et al*. 2011; Jobling *et al*. 2012; Hamre *et al*. 2013), is likely to be the major factor accounted for the poor performance of early *H. kuda* juveniles culture.

Although the culture technique and optimum feeding regime are likely to be species specific, juvenile seahorses are feeding almost exclusively on copepods in the wild (Castro *et al*. 2008; Olivotto *et al*. 2011; Yip *et al*. 2015). Indeed, the captive rearing of *H. kuda* juveniles is commonly relied on wild zooplanktons which mainly consist of copepods during the first week of culture (Job *et al*. 2002; Choo & Liew 2006; Celino *et al*. 2012). However, such practice could risk introduce some harmful pathogens into the culture tank (Buen-Ursua *et al*. 2011; LePage *et al*. 2015). Furthermore, many studies have indicated that the intra- and inter-specific variations including life history, geographical distribution and feeding ability may also affect the specific feeding requirements and culture conditions of juvenile seahorses (Wilson & Castro 2010; Olivotto *et al*. 2011; Jobling *et al*. 2012; Zhang *et al*. 2015b).

1.2 Problem statements

Despite numbers of studies on the culture and morphological development of seahorses have been reported (Mi *et al*. 1998; Job *et al*. 2002; Choo & Liew 2006; Celino *et al*. 2012), a significant gap in the fundamental knowledge of morphological development and feeding ability of juvenile seahorse is still hampering the formulation of an optimum feeding regime and culture technique to enhance the survival and growth of *H. kuda* early juveniles. A critical transition from endogenous to exogenous feeding occurs upon their released from the paternal brood pouch may have responsible for the mass mortalities among *H. kuda* juveniles during the first 7–10 days (Sifa & Mathias 1987; Lin *et al*. 2006; Celino *et al*. 2012).

1.3 Research hypothesis and objectives

To potentially improve the survival and growth of early *H. kuda* juveniles in captivity, comprehensive studies were conducted to resolve the feeding requirements of *H. kuda* juveniles specifically during the critical phase of first 10 days. The null hypothesis was that *H. kuda* juveniles had a fully developed feeding ability upon birth and could be cultured *en masse* using the selected live foods. In order to assess the validity of hypothesis, morphological developments of the feeding apparatus and digestive system in *H. kuda* juveniles were evaluated via microscopic, histological and biochemical approaches, while the effects of selected live foods on the survival and growth of *H. kuda* juveniles during the critical life phase were determined. The specific objectives of this study were:

1. To evaluate the mouth structure, external morphology, prey capturing behaviour and ontogeny of alimentary tract in *Hippocampus kuda* juveniles during the first 10 days.
2. To investigate the ontogeny of proteolytic digestive enzymes activities in *Hippocampus kuda* juveniles and the changes of their digestive enzyme activities before and after feeding a combination of selected live foods.
3. To determine the effects of different feeding regimes that consist of rotifers *Brachionus* sp., brine shrimps *Artemia* sp. and cyclopoid copepods *Oithona simplex* on the survival, growth and prey consumption of *Hippocampus kuda* juveniles during the first 10 days.

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