

ผลของอาหารต่อการเติบโตและการพัฒนาของอวัยวะสร้างเซลล์สืบพันธุ์ม้าน้ำ *Hippocampus* sp.



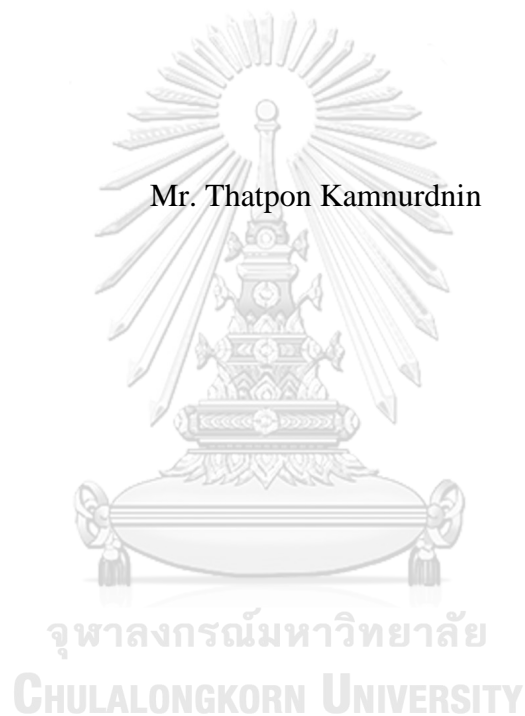
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EFFECTS OF FOOD ON GROWTH AND GONADAL DEVELOPMENT OF SEA
HORSE *Hippocampus* sp.

Mr. Thatpon Kamnurdnin



A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Marine Science
Department of Marine Science
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บทคัดย่อ

ม้าน้ำ (*Hippocampus* sp.) เป็นปลาสวยงามที่มีความสำคัญทางเศรษฐกิจ ผลจากการส่งออกม้าน้ำเชิง
 อุตสาหกรรม การทำประมงเกินขนาด และการรุกรานแหล่งที่อยู่อาศัย ส่งผลให้ประชากรม้าน้ำในธรรมชาติลดลง
 ดังนั้นการเพาะเลี้ยงจึงอาจเป็นวิธีที่ช่วยเพิ่มจำนวนประชากรม้าน้ำได้ ซึ่งข้อมูลด้านอาหารต่อการเติบโต และการ
 พัฒนาของม้าน้ำ มีความจำเป็นต่อการเพาะเลี้ยงที่มีคุณภาพ การศึกษานี้จึงมุ่งเน้นผลของอาหารต่อการเติบโตและ
 การพัฒนาของอวัยวะสืบพันธุ์ของม้าน้ำ (*Hippocampus* sp.) ตั้งแต่แรกเกิดถึงอายุ 31 วัน ม้าน้ำถูกเลี้ยงภายใต้
 ระบบเพาะเลี้ยงในภาวะแวดล้อมเหมาะสม ณ โรงเพาะฟักและอนุบาลสัตว์น้ำ ศูนย์ชีววิทยาทางทะเล จังหวัดภูเก็ต
 โดยใช้อาหาร 4 ชนิด คือ ไรทะเล (*Artemia* sp.) กุ้งเคยจากธรรมชาติ (*Mysis* spp.) ลูกกุ้งขาว และโคฟีพอดเสริม
 สารอาหาร แบ่งการศึกษาเป็น 2 ส่วน การศึกษาส่วนแรกคือ การเติบโตและอัตราการรอด จากการศึกษาพบว่า ม้าน้ำที่
 ได้รับอาร์ทีเมียเป็นอาหาร ตายหมดในช่วง 10 วันของการทดลอง ม้าน้ำหนามขอที่ได้รับกุ้งเคยเป็นอาหารมีอัตรา
 รอดและเติบโตสูงสุด รองลงมาคือลูกกุ้งขาว และโคฟีพอดเสริมอาหาร ตามลำดับ การศึกษาส่วนที่สองคือการ
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 ระยะเวลาพัฒนาของเซลล์ไข่ในกลุ่มที่ได้รับกุ้งเคย สั้นกว่าลูกกุ้งขาวและ โคฟีพอดเสริมอาหาร ทั้งนี้ในม้าน้ำ
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 พัฒนาของอวัยวะสืบพันธุ์ของม้าน้ำหนามขอสอดคล้องกับอัตราส่วนปริมาณของกรดไขมันในกลุ่มกรดไขมันไม่อิ่มตัวสูง
 (highly unsaturated fatty acid) ที่พบสูงที่สุดในกุ้งเคยธรรมชาติ ลูกกุ้งขาว และโคฟีพอดเสริม
 สารอาหาร จากผลการทดลองสามารถสรุปได้ว่ากุ้งเคยธรรมชาติและลูกกุ้งขาว เป็นอาหารที่เหมาะสมต่อการ
 เติบโตและการพัฒนาของอวัยวะสร้างเซลล์สืบพันธุ์ของม้าน้ำ *Hippocampus* sp. ซึ่งสามารถนำไปเป็นแนวทาง
 เพื่อพัฒนาระบบการเพาะเลี้ยงม้าน้ำได้ในอนาคต

คำสำคัญ ม้าน้ำ (*Hippocampus* sp.) ชนิดอาหาร อัตราการเติบโต การพัฒนาของอวัยวะสร้างเซลล์
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THATPON KAMNURDNIN: EFFECTS OF FOOD ON GROWTH AND GONADAL DEVELOPMENT OF SEAHORSE *Hippocampus* sp.. ADVISOR: SUPANUT PAIROHAKUL, Ph.D., CO-ADVISOR: PARICHART LAKSANAWIMOL, Ph.D., 70 pp.

ABSTRACT

Hippocampus sp. has received attention as a source of aquarium and ornamental trade. Seahorses have been threatened from non-target fisheries, habitat loss and traditional Chinese medicine (TCM). In response to concern about a decrease of population in the wild, an aquaculture of this species may provide the seahorse numbers for the commercial demands. However, one of the critical problems in aquaculture is the proper dietary for growth and their development. Therefore, this study was conducted on different types of preys on survival rates, growth rates and gonadal development in *Hippocampus* sp. during the day of birth until the 31 day after birth (DAB). The newborn *Hippocampus* sp. was reared under the optimal environmental conditions at the hatchery of Phuket Marine Biological Center (PMBC). The feeding treatments provided to newborns *Hippocampus* sp. were *Artemia*, wild *Mysis* spp., shrimp larvae and enriched copepod. The study conducted into 2 aspects: growth rates and survival rate, and gonadal development. In the case of *Hippocampus* sp. growth, none of the *Artemia*-fed group survived. The *Mysis*-fed group has the highest in growth rates and survival rates followed by the shrimp larvae-fed group and enriched copepod-fed group. For gonadal development, only ovaries of *Hippocampus* sp. were observed under histological level. The duration of oocyte growth in *Mysis*-fed group was shorter than the shrimp larvae-fed group and the enriched copepod-fed group, respectively. The result of growth rates and gonadal development are also related to highly unsaturated fatty acid (HUFA) contents in preys, which was observed high contents HUFA in *Mysis* spp., shrimp larvae and enriched copepod, respectively. The results of this study demonstrated that the *Mysis* spp. and shrimp larvae could be provided as an optimal prey for *Hippocampus* sp. growth and gonadal development. Therefore, this feed could be applied to improve the seahorse aquaculture in the future.

Keyword *Hippocampus* sp., Preys, Growth, Gonadal development, Diet composition

Department: Marine Science

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CHAPTER 1

INTRODUCTION

Statement of Problem and Significance of Research

Seahorse is one of the important marine fish for economical trade and can be exported for the traditional Chinese medicine material which can lead to rapid decreasing of the population. In response to the concern about the declining of the seahorse population in the wild, an aquaculture of this species may provide the seahorses for the commercial demands. Several studies have been conducted to improve seahorse growth and survival rates in several species. However, one of the critical problems in seahorse aquacultures is the improper and imbalance of food nutrition for their good health, increasing in juvenile mortality and declining in growth rate. Therefore this study could demonstrate the effects of different types of feed diets for growth rate, survival rate and gonadal development in order to improve the production efficiency in the seahorse aquaculture, which was limited study.

Research Hypothesis

The wild *Mysid* and the post-larvae shrimps will be the most suitable prey in this experiment. The seahorse fed with the wild mysid and the post-larvae shrimp may provide higher survival rate, growth rate and gonadal development compared with the enriched copepods.

Objectives

To study the effect of food on growth and gonadal development of the seahorse (*Hippocampus* sp.).

Applications

The outcomes may contribute to an understanding about the different types of live preys which can affect survival rates, growth rates and gonadal development of the seahorse. Moreover, the knowledge of growth and gonadal development from this study can further apply in seahorse aquaculture development and maintain the seahorse population in the wild.



CHAPTER 2

LITERATURE REVIEW

Biology of the seahorse Hippocampus sp.

According to the reported by Lourie et al. (2004), *Hippocampus* sp., is a marine teleost species belonging to phylum Chordata; order Syngnathiformes; family Syngnathidae. The seahorse is usually found along the shallow tropical and temperate marine ecosystems. They mainly inhabit around coral reef area, living as epibenthos on large seaweed or even living on mangrove root and seagrasses (Lourie et al., 2004). The newborn seahorse lives as a planktonic larvae floating along the seawater current. Seahorse usually hold the object under water with their prehensile tail such as coral limb and seagrasses leaf. They can swim slowly and poorly using their dorsal fins and pectoral fins. The long snout is used for sucking up their food. Their eyes can independently move from each other (Lourie et al., 2004).

Seahorse is a monogamous species during a breeding season. The couples form pair bonds throughout the breeding season. The female places their eggs into the male's pouch and then male becomes "pregnant" (Fig 2-1)



Figure 2-1 Male *Hippocampus* sp. with brood pouch becomes pregnant in a breeding season.

According to the *Hippocampus* sp. in this study, the seahorse has an angle positioned-head to their body. The bony plate covers all their trunks and prehensile tails. The thorny seahorse has shown some variety of their color including pale yellow and white. The distinctive characteristics of the seahorse are the four or five sharp spine coronets, well-developed long spines with black tips on their body, the distinctive sharp long-spine on their noses and eyes, a single cheek spine, the snout length is as long as head length, and 15 dorsal fin rays and 18 pectoral fin rays (Fig 2-2).

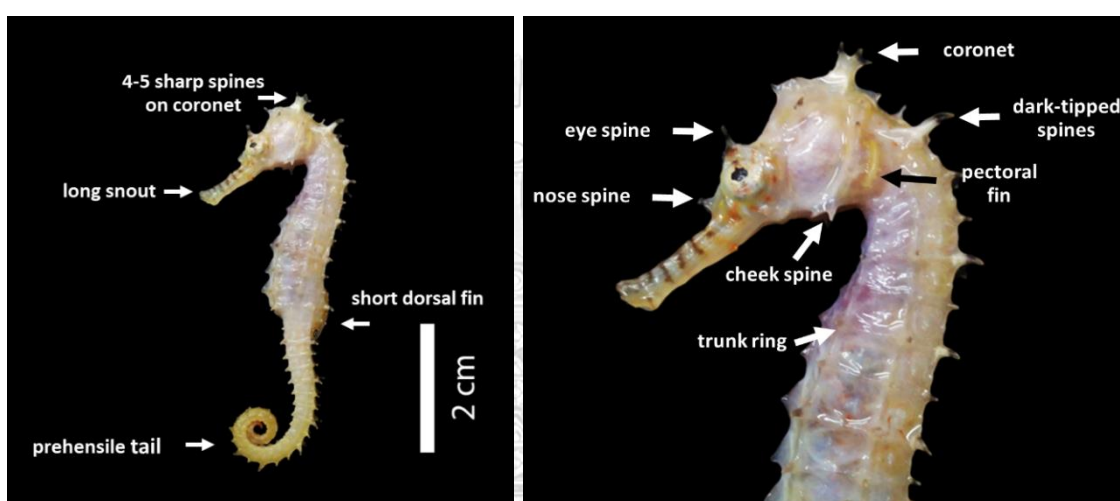


Figure 2-2 The distinctive characteristics of the seahorse of adult *Hippocampus* sp. show four sharp spine coronets, well-developed long spines with black tips on their body, the distinctive sharp long-spine on their noses and eyes, a single cheek spine, the snout length is as long as head length, and 15 dorsal fin rays and 18 pectoral fin rays.

Feeding Habits of the Seahorse

Seahorses are ambush predator that can wait for their small preys using their tubular snouts. Wild seahorses can feed on several species of preys that float on the surface of seawater. The study of the lined seahorse (*Hippocampus erectus*) feeding displayed some different types of their wild preys including crustacean, molluscs, polychaetes and so on. However, most of the preys in their guts were amphipods and copepods, respectively (Teixeira and Musick, 2001). Similarly, the study by Woods, 2002 showed that the top three major preys of the large-bellied seahorse (*Hippocampus abdominalis*) are crustacean, amphipods and decapods, respectively.

Threats to the Seahorse

The seahorses were threatened from over-exploitation, non-target fishing gear and the degradation of their habitats (Perry et al., 2010). Seahorses are also provided to the international trade both as dry specimens to use in particular traditional Chinese medicine (TCM) or as live specimens for ornamental trades and as aquarium fish (Vincent, 1996).

Dried seahorse trade were recorded about at least 45,000 kg annually around the Asia during 1993- 1995 (Vincent, 1996). According to the government document reported by Anonymous (2001), the seahorse demand has been continuously increased every year. The data from Hong Kong and Taiwan government during 1998-2004 period showed the large amount of dried seahorse approximately 15,400 kg was exported from Thailand. Most of seahorse exploitation can result in declining of seahorse population in the wild at the rate about 15-50% over 5 year. Some species were traded in Thailand and the nearby area included at least five species e.g. *H. kelloggi*, *H. kuda*, *H. spinosissimus*, *H. trimaculatus* and, and *H. histrix*. (Perry et al., 2010).

Since wild seahorse population rapidly decreased over the year. Thus the seahorse species were listed on the IUCN Red List in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The CITES listing are used for monitoring the seahorse population in the wild. Management procedure of export especially the commercial international trade could ensure that the export do not affected the wild seahorse population. (www.unepwcmc.org/species/sca/scs.htm).

Aquaculture of the Seahorse

Recently, the attention of the seahorse aquaculture is resulted from overexploitation of seahorses in the wild population. There are continual researches about the seahorse aquaculture to provide the optimal conditions in the captive condition. The seahorse culture goal is to increase survival rates and growth rates. However, each seahorse species requires different conditions. There have been several studies conducted in various factors particular in the appropriate dietary for survival rate, growth rate and seahorses development. (Job et al., 2002; Lin et al., 2007; Murugan et al., 2009; Woods and Valentino, 2003).

In the captive conditions, live preys e.g. copepod (Murugan et al., 2009), rotifers (Otero-Ferrer et al., 2010), mysids (Woods, 2005) and *Artemia* (Woods and Valentino, 2003) were applied to the culture of seahorse. The seahorse fed with different preys showed different growth rate. During early stage of growth, *Hippocampus trimaculatus* preferred copepods (Murugan et al., 2009), (Sheng et al., 2006). The *H. trimaculatus* juveniles fed by the copepods showed high survival rate (Murugan et al., 2009). The study of *H. trimaculatus* (Murugan et al., 2009) showed the similar result to the *H. guttulatus* juvenile (Palma et al., 2014). In the case of the seahorse fed by rotifer, the poor survival rates and biometric values were recorded (Otero-Ferrer et al., 2010). In addition to the live brine shrimp (*Artemia salina*), the enriched-*Artemia* were also often fed to the juvenile seahorse. The enriched-*Artemia* fed in *Hippocampus abdominalis* had 100% survival rate and the feed also promotes a good growth rate (Woods and Valentino, 2003). On the other hand, the *H. guttulatus* juvenile fed with the enriched-*Artemia* had all died in 120 hours after their first meal (Palma et al., 2014).

Reproductive System and Gonadal Development of Seahorse

There were some previous studies of the gonadal structure and gonadal development in Syngnathidae (Begovac and Wallace, 1988; Piras et al., 2016); however, there were a few researches conducted in the seahorses. Nevertheless, the results of gonadal development may provide the estimation of reproductive period and gamete quality (Lin et al., 2006). These knowledges can be applied for increasing in the efficiency of seahorse aquaculture

Ovarian Structure and Differentiation

In Syngathids, the ovarian tissue undergoes a sequence of morphological transformation during each reproductive cycle and maturation. The egg productions are the asynchronous type (Wallace and Selman, 1981). Asynchronous type of ovarian development is exhibited by the species which produce multiple spawns that present all classes of the oocytes in their ovary (Fig 2-3). In seahorse, a pair of ovary is located in the posterior region of the coelomic cavity. At the external area of ovary, the muscular wall is externally covered with the epithelium, called mesothelium, which is connected to the mesovarium. The entire ovarian cavity is lined with luminal epithelium. Follicle lamina is located between the muscular wall and luminal epithelium where germ cells can develop at the germinal ridge. As mentioned earlier, follicles are developing toward the center of the follicular sheet (Fig 2-3).

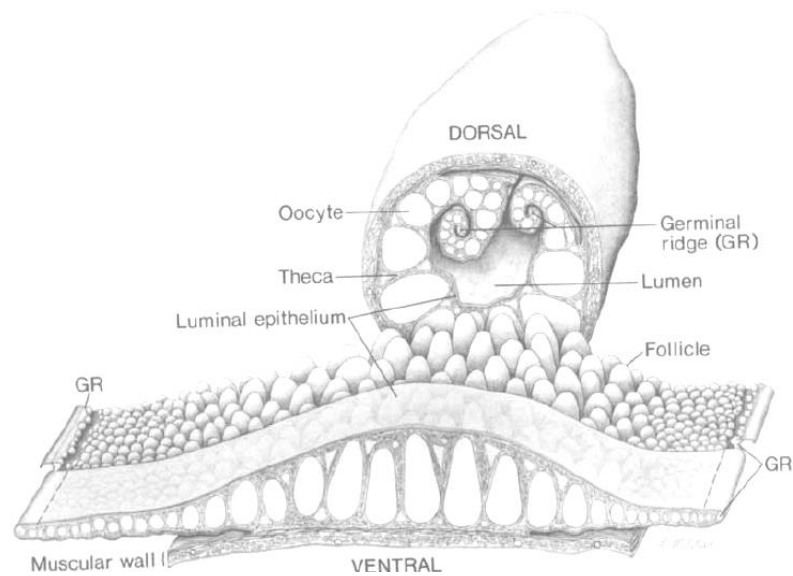


Figure 2-3 Ovarian structure of the seahorse. The ovary is externally covered with the muscular wall. The entire ovarian cavity is lined with luminal epithelium. Follicle lamina is located between the muscular wall and luminal epithelium where germ cells can develop at the germinal ridge. (Selman, Wallace et al. 1991)

In each ovary, the ovarian follicles are produced in the germinal ridge (Selman et al., 1991). The germinal ridge contains different stages of germ cells including oogonia, meiotic prophase oocyte and larger oocytes (Selman et al., 1991) (Fig 2-4). The stages of oocyte development could be divided into three developmental stages followed the criteria by Carmen Uribe et al. (2012) was showed as Fig 2-5: oogonia proliferation stage, primary growth stage, and secondary growth stage. According to the oocyte classification by Carmen Uribe et al. (2012), oogonia proliferation stage exhibits scattered oogonia throughout the germinal epithelium. Primary growth stage, this can divided into 4 stages: chromatin nucleolus stage, multiple nucleoli stage, perinucleolus stage, and oil droplets and cortical alveoli stage. Chromatin nucleolus stage exhibits an enlarge nucleus with basophilic ooplasm. Oocyte then increases in size and they contain multiple nuclei which are distributed in germinal vesicle and considered as multiple nucleoli stage. Oocytes in perinucleolar stage contain many nucleoli disperse around the nuclear envelope. Oocyte can then differentiate into oil droplets and cortical alveoli stage. The oil droplets appear around the germinal vesicle. For secondary growth stage, this can be divided into early and late secondary growth stage oocyte. Early secondary growth stage can be found that the yolk vesicle begin to

fuse and form yolk globule while the late secondary growth stage of oocyte exhibits large region of yolk globules in the ooplasm.

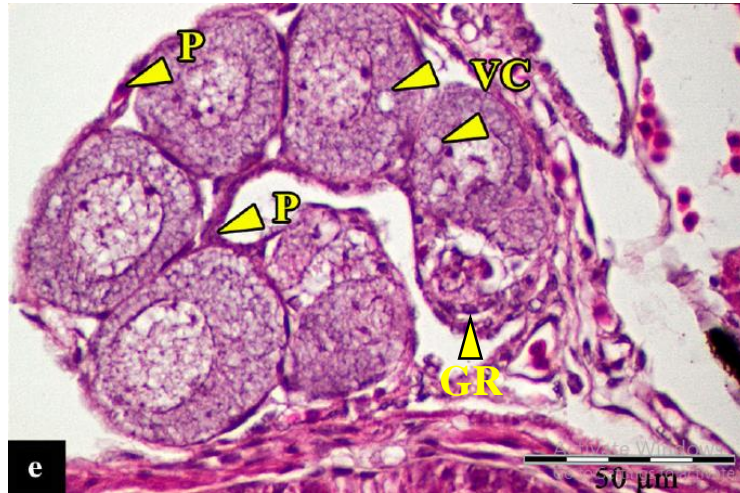


Figure 2-4 The ovary is surrounded by the peritoneal membrane. In the ovarian compartment, the ovarian follicles are produced in the germinal ridge. *P* peritoneal membrane, *VC* fat vacuoles, *GR* germinal ridge (Selman et al., 1991).

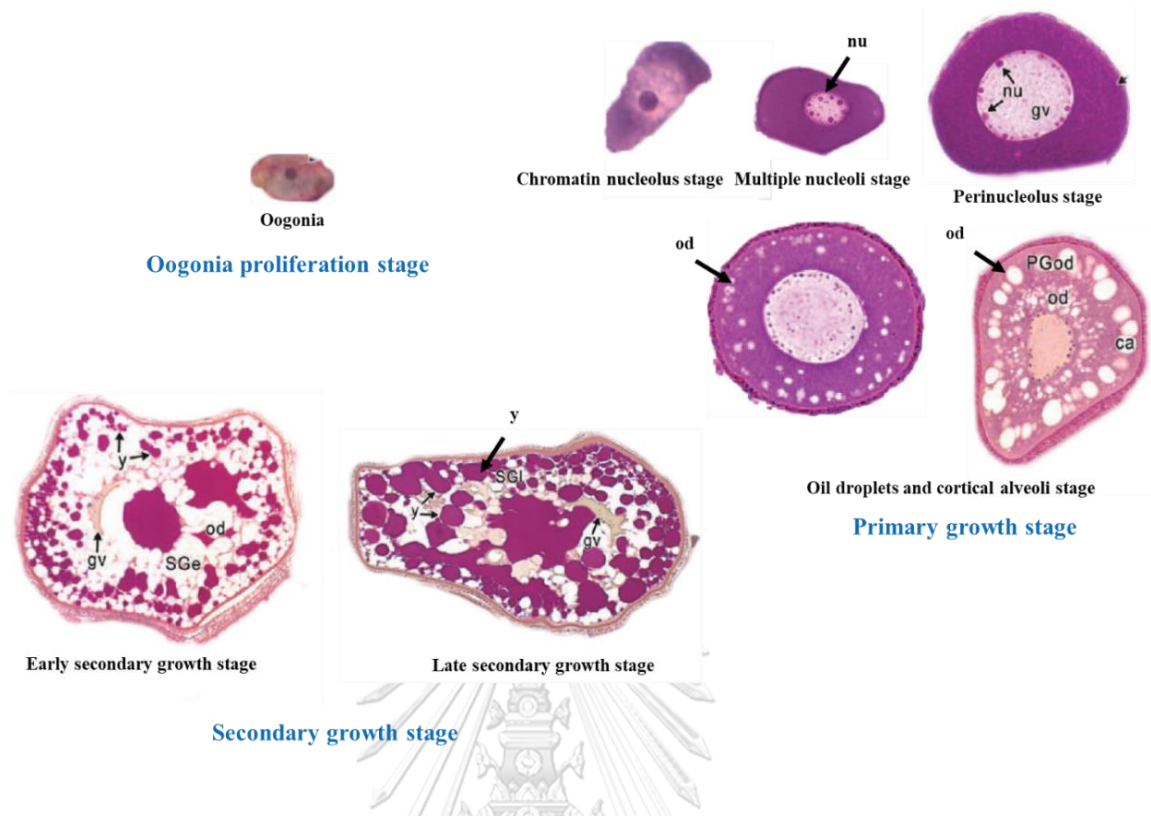


Figure 2-5 Oocyte classification in fish include 3 main stages; oogonia proliferation, primary growth stage, and secondary growth stage following Carmen Uribe et al. (2012) *nu* nucleolus, *gv* germinal vesicle, *od* oil droplets, *PGod* primary growth oil droplets stage, *ca* cortical alveoli, *y* yolk, *SGe* secondary growth in early stage.

Testicular Structure and Differentiation

In male seahorse, paired testes are tubular organ which adhere to the abdominal cavity. Each testis is characterized by testicular lumen and thin testicular wall. Testicular wall is surrounded by two tissue layers; tunica albuginea and germinal epithelium. The germinal compartment overlays the end of the testes where the germ cells are formed and developed to be the spermatocytes (Fig 2-6).

The seahorse testis is an unrestricted type, where spermatogonia are arranged along the lengths of lobules (Laksanawimol, 2004; Piras et al., 2016). According to the reported by Carcupino et al. (1999) and Piras et al. (2016) , in syngnathids testes, spermatogonia and primary spermatocyte are contained in spermatocyst. Spermatogonia has mitotic division and give rise to spermatocyte. Primary spermatocyte has meiotic division which is become secondary spermatocyte. The secondary spermatocyte then develops to spermatids and spermatozoa which are founded in the testicular lumen (Fig 2-7)

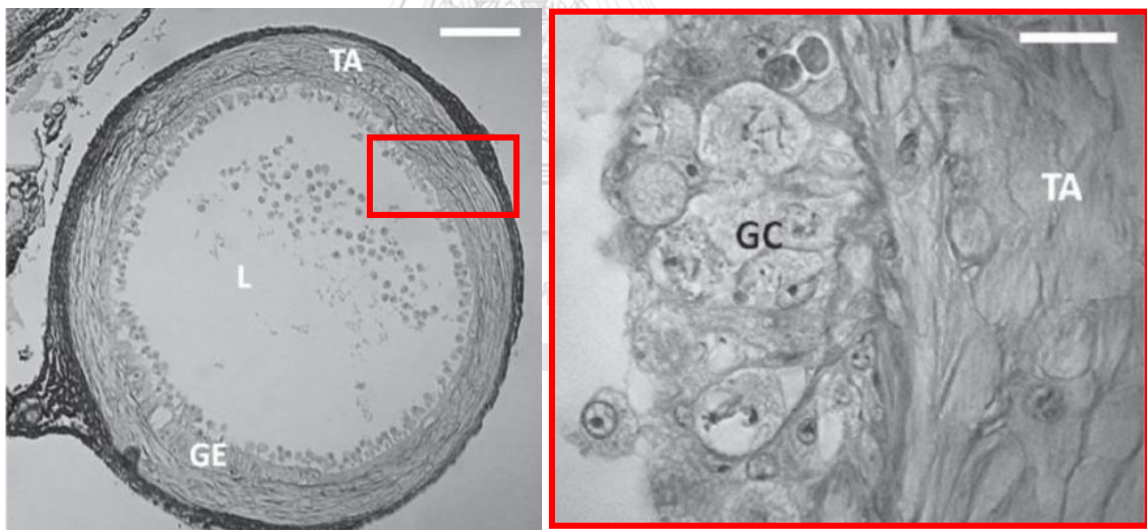


Figure 2-6 The testis is characterized by testicular lumen and thin testicular wall. Testicular wall is surrounded by two tissue layers include of tunica albuginea and germinal epithelium. The spermatogonia and spermatocyte formed by germ cell ; *GE* germinal epithelium, *TA* testicular albuginea, *GC* germ cell, *L* lumen (Piras et al., 2016)

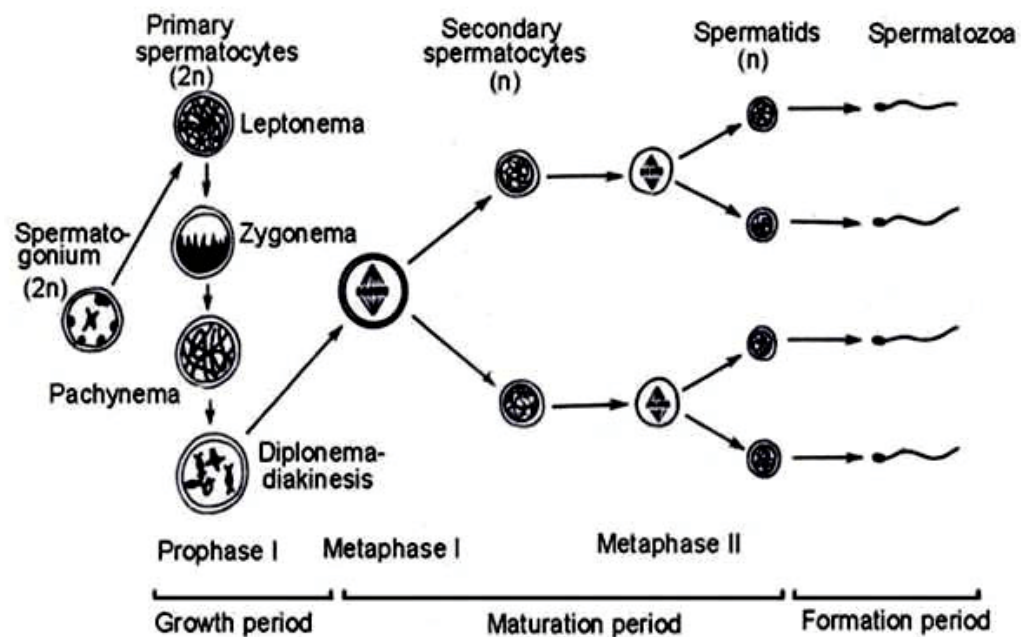


Figure 2-7 Scheme of spermatogenesis in fish. Spermogonia has mitotic division and give rise to spermatocyte. Primary spermatocyte has meiotic division which is become secondary spermatocyte. The secondary spermatocyte then develops to spermatids and spermatozoa (Gomelsky et al., 2011).

Diet Composition on Growth and Gonadal Development

Growth performances, survival rates and early developments of fish juveniles in aquaculture are related to feeding strategies and nutrient requirements (Izquierdo, 1996; Olivotto et al., 2008; Regost et al., 2003). Thus, several studies often focus on essential dietary composition especially protein and fatty acid contents in feed for the early development of fish (Izquierdo, 1996; Izquierdo et al., 2001; Palma et al., 2008).

There were several studies on fatty acid compositions on the growth, survival rates and the development of some teleosts. In case of the fish growth, the study of Bell et al. (1985) showed that weight loss occurred in the turbot (*Scophthalmus maximus*) fed with the deficient polyunsaturated fatty acid diet (PUFA). Furthermore, fatty acid in the tissues of the turbot decreased when the fatty acid in the diet decreased. The study of Lochmann and Gatlin (1993), the juvenile red drum (*Sciaenops ocellatus*) fed with essential fatty acids-deficiency (EFAs) diet had high mortality rates and low weight

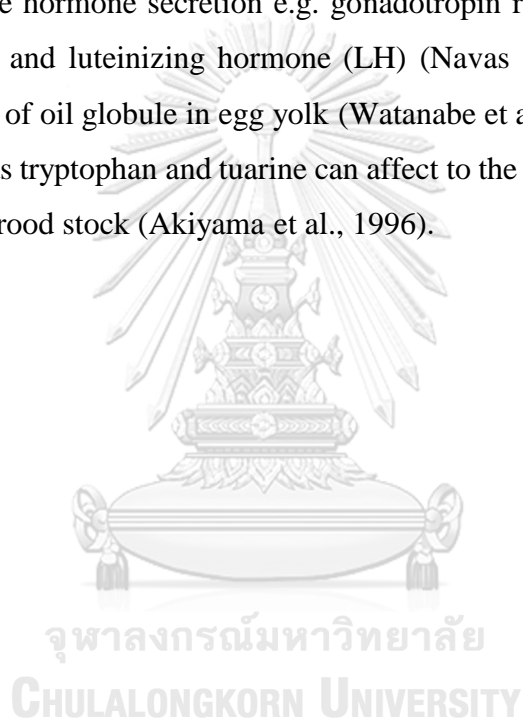
gain. On the other hand, the growth and survival rates of the juvenile red drum increased when they fed with highly unsaturated fatty acid (HUFA) diets.

In terms of gonadal development, the study of fatty acid commonly conducted in the fish brood stock. e.g. the study in male gilthead seabream brood stock have been shown that high fatty acid composition can increase gonad development. Moreover, the study of Goetz et al. (1991) and Sorbera et al. (1998) suggested that the fatty acid composition required for developing oocytes, especially during vitellogenetic stage. For hormonal regulation, an increasing in arachidonic acid (ARA) resulting in testicular steroidogenesis in goldfish which can further regulate androgen production (Wade and Van Der Kraak, 1993).

Fatty acids are important nutrient as the sources of energy in early embryonic development. Moreover, fatty acids can also function as a component in phospholipid molecules in fish membrane (Rainuzzo, 1993). Some essential fatty acid particular n-3 highly unsaturated fatty acids (n-3 HUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were found in high concentration in their nervous system and in tissues which can result in growth regulation (Otero-Ferrer et al., 2010), larvae survival rates, membrane fluidity, lipid metabolism and immune functions in fish (Watanabe et al., 1984). Moreover, the previous study reported by Ji et al. (2011) suggested that HUFA affected the activity and gene expression of lipid metabolism related enzymes such as glucose-6-phosphate dehydrogenase (G6PDH) and malate dehydrogenase (MDH). G6PDH and MDH can provide NADPH and NADH that are essential for lipid synthesis, lipoprotein lipase, which hydrolyzes triglycerides in circulating lipoproteins. Moreover, they also affect the transcription of peroxisome proliferator-activated receptors (PPARs), which play key roles in the catabolism and storage of fatty acids.

In addition to the protein content in the diet, an increasing in the optimal protein content could also improve growth rates and feeding efficiency which was reported in the Atlantic halibut (*Hippoglossus hippoglossus*) (Aksnes et al., 1996). The growth rates and survival rates were observed in the tilapia juvenile (*Sarotherodon mossambicus*), the post-molting stage of the Atlantic salmon (*Salmo salar*) and the

juvenile flounder (*Paralichthys olivaceus*) (Jauncey, 1982; Lee et al., 2000; Refstie et al., 2004). On the other hand, the study of Kaushik et al. (2004) showed different results in the European seabass (*Dicentrarchus labrax*) growth. The European seabass showed no significant differences on growth when they fed with different protein contents. Furthermore, protein contents in fish diet also play an important role in regulation of the oocyte maturation. The study of different protein content diet in tilapia (*Oreochromis niloticus*) showed that increasing in protein content reached the oocyte to the post-vitellogenic stage (Gunasekera et al., 1995). However, low protein intake may affect to some hormone secretion e.g. gonadotropin releasing hormone (GnRH) (Kah et al., 1994) and luteinizing hormone (LH) (Navas et al., 1997) and cause an increasing number of oil globule in egg yolk (Watanabe et al., 1984). Moreover, some amino acids such as tryptophan and taurine can affect to the gonadal maturation in both male and female brood stock (Akiyama et al., 1996).



CHAPTER 3

METHODOLOGY

The juvenile *Hippocampus* sp. were reared in the marine hatchery at Phuket Marine Biological Center (Phuket, Thailand) under the optimal conditions. The juvenile seahorses were divided into to 4 experimental groups and were fed with different 4 experimental feedings. The juvenile seahorses were then randomized selected for examination the effect of different food sources on growth and the gonadal development.

Feeding Preparations for Juvenile Hippocampus sp. Treatments

The juvenile brine shrimps (*Artemia salina*) and the captive copepods were derived from the hatchery at Phuket Marine Biological Center (PMBC, Thailand). *Artemia* juveniles with 10-12 day were hatched from the *Artemia*-cyst (Eggs/American Eagle®). In the case of the captive copepods, they were enriched with fish oil, vitamin C and vitamin E for 3 hours. The wild krill from the off-shore near the PMBC were captured by plankton net with the 200 µm mesh size and were maintained in the tank for feed the juvenile seahorse. In the case of the post-larvae shrimps (*Litopenaeus vannamei*) from Siam Andaman Seabass farm (Phuket, Thailand), the larvae were applied in the experiment within 13-17 days after hatching. Wet weights of each feed were recorded and provided to each experimental unit at the amount of 12% of the seahorse weight (Woods, 2005).

Brood stock selection of Hippocampus sp.

Each brood stock was selected from the healthy adult *Hippocampus* sp. seahorse cultures at Phuket Marine Biological Center (PMBC, Thailand) which has 15-17 cm in height (Masuda et al., 1985) as shown in Fig 3-1.



Figure 3-1 The healthy *Hippocampus* sp. brood stock selection. Female seahorse (A) and male seahorse (B) distinguished a brood pouch; *BP* brood pouch.

Three pairs of parental *Hippocampus* sp. seahorses were reared in round fiber glass tanks with volume of 500 liters capacity. The parent seahorses were kept under the optimal conditions: temperature at 28 ± 0.5 °C, salinity at 33 ± 0.5 ppt, DO at 6.5 ± 0.5 mg/l and pH at 8.33 ± 0.5 . The animals were reared under photoperiod of 12:12 hour and the amount of wild krill were fed at the weight of 12% of their initial individual weight of each day (Woods, 2005).

After mating, the pregnant males shown the dark grey color with the large brood pouch. The individuals of the pregnant male were then separated into nursery tanks with maintaining the same rearing condition as described earlier. After 3 weeks of pregnancy, the juvenile seahorses were released. Each male brood stock provides around 30-60 newborn seahorses. Finally, the male brood stock was removed from the tank.

The Juvenile Hippocampus sp. Rearing and Sampling

In each of the experiment, the juveniles *Hippocampus* sp. were reared under the optimal conditions as described in the previous subtopic. Individuals of juveniles *Hippocampus* sp. were fed at the amount of 12% of their initial individual weight of each day (Woods, 2005). The feeding behaviors were also observed during the feeding times. The wastes were cleaned up after the juvenile fed all the preys within 3 hours.

The 108 newborns *Hippocampus* sp. were used in the study divided into; 93 individuals for experimental treatment, and 15 individuals for fatty acid and protein analysis. Because of the small numbers of newborn seahorse from each brood, the seahorse samples were obtained from 2 repetitions of broods from the same paired of seahorse. The first brood of seahorses (n = 63) were divided into 4 experimental treatment; *Artemia*-fed group, *Mysis*-fed group, shrimp larvae-fed group and enriched copepod-fed group. The 3 juveniles were randomly sampled every 3 days since 8 DAB until 17 DAB. In the case of the second brood of seahorses (n=45), they were divided into 3 experimental treatment; the *Mysis*-fed group, the shrimp larvae-fed group and the enriched copepod-fed group. The 3 juveniles were randomly sampled every 3 days since 20 DAB until 31 DAB. The experimental design can be shown in Figure 3-2

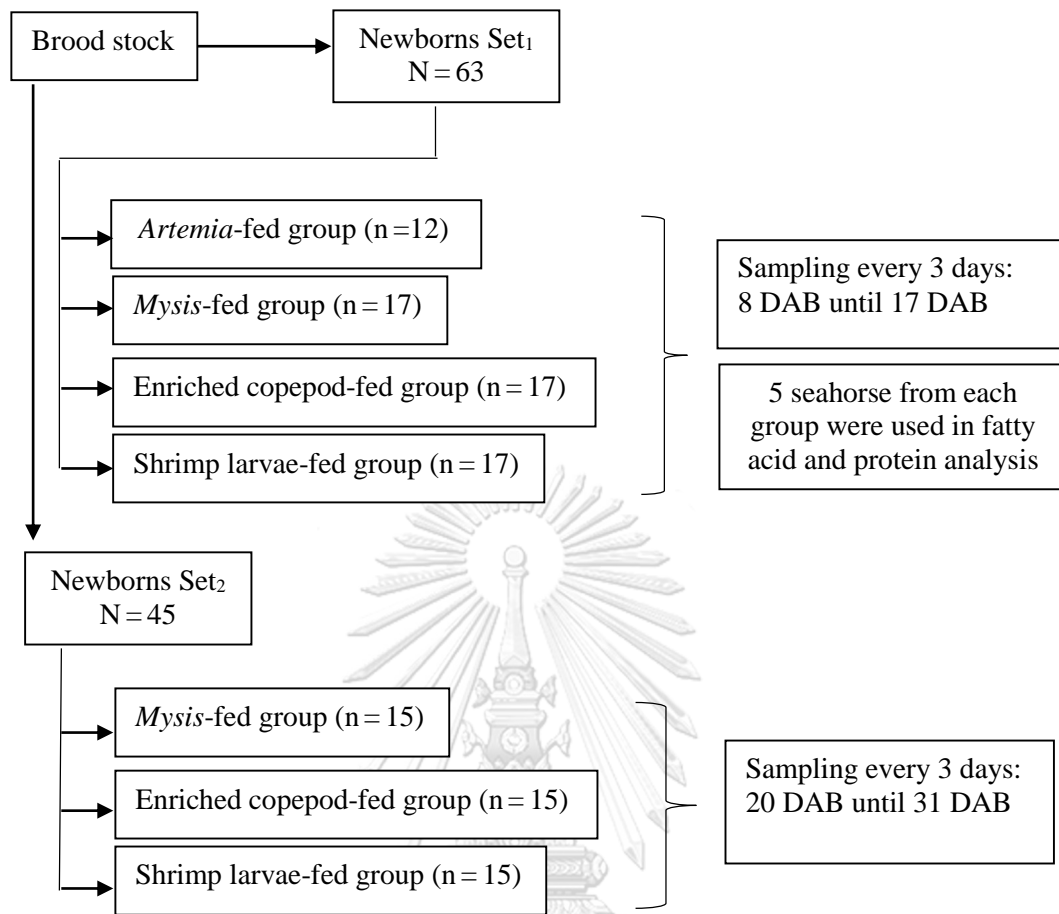


Figure 3-2 The experimental design of the juveniles *Hippocampus* sp. sampling in each the experimental treatment: *Artemia*-fed group, *Mysis*-fed group, enriched copepod-fed group, shrimp larvae-fed group; *DAB* day after birth.

The total lengths of the *Hippocampus* sp. samples were individually and daily measured followed the method by Lourie (2003). Body wet weights were also measured for growth analysis. The live seahorse samples were knocked by rapid cooling followed the method by Wilson et al. (2009) with seawater under 0°C temperature. After that, the samples were preserved in Davidson's fixative for 36 hour and move to 70% ethanol (The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science in accordance with the guide for the care and use of laboratory animal prepared by Chulalongkorn University Protocol Review No. U1-02042-2558).

Growth analyses of the Juvenile Hippocampus sp.

The total lengths of the 3 juvenile *Hippocampus* sp. samples were individually measured followed by Lourie (2003) daily and the body wet weight were also measured for growth analysis as mentioned earlier.

The percentage of specific growth rate (%SGR) was also calculated according to (Ricker, 1958) as shown in the following equation.

$$\text{SGR} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \times 100$$

Where the W_1 is an initial wet weight at time t_1 while W_2 is a final wet weight at time t_2

The condition factor (CF) was calculated for each seahorse treatment as shown in the equation below.

$$\text{CF} = \frac{\text{Wet weight}}{\text{Total length}} \times 100$$

The SGR can be utilized for an estimation of the effective of growth (percentage per day) during the experiment growth period. While the CF can be applied for an analysis in the external morphology resulted from different food and sex. Finally, the survival rates were estimated at the end of the experiment to determine the effect of diets.

The histological Process of Juvenile Hippocampus sp.

The *Hippocampus* sp. samples were cut cross the gonad position at the first dorsal fin ray position followed the method of Novelli et al. (2015). The samples were preserved in Davidson's fixative for 36 hours. The fixed *Hippocampus* sp. samples were processed followed the standard histological technique by Humason (1962). The tissues samples were serially dehydrated in 70%, 95% and 100% ethanol for dehydration process for 2 hours in each step. The sampled tissues were incubated in the xylene. After that, the samples were embedded with the Paraplast wax (Leica®). The paraffin blocks were then sectioned at 5 μm thickness and routinely stained with Hematoxylin and Eosin (H&E) (Presnell et al., 1997). The gonadal development of seahorse were examined using light microscope and photomicrography with Leica DM750. Characteristics of cells, gonadal compartments and gonadal differences were

observed. Moreover, the juvenile seahorse gametes were measured using the Leica light microscope with Leica LAS4.9.

The Diet Composition of Juvenile Hippocampus sp. and Feeding

According to the fatty acids procedure, the solid samples was blended by the blender and then transfer of 3 g into a tube. The ratio 2:1 of dicloromethane to methanol (%v/v) was added to a volume of 12 ml. The samples were vortexed and stood for an hour. The samples were then filtrated with Whatman no.1 and 0.1 M KCl were added. After that, the samples were vortexed and centrifuged and then keep the lower solution to perform methylation. The samples were added with NaOH-methanol and then heat at 100 °C and cool down in room temperature. The volume of 2 mL of BF₃- Methanol was added. The samples were heated again, then the Hexane and saturated NaCl were added in the tube. The samples were vortexed and centrifuged for 5 min. Finally, the upper phase oil in vial were analyzed under gas chromatography mass spectrometry (GC –MS) process as shown in Fig 3-3. According to the protein procedure followed by Latimer (2012), the 1 g of samples were added the Kjellab catalyst. The samples were then added 15 ml H₂SO₄. After that, the samples were digested at 420 °C for 60 minutes. The samples were then processed in the distillation and then titrate with 0.1 HCl. Finally, the samples protein were calculated (Fig 3-3).

Statistical analyses

Variations between the *Artemia*-fed group, *Mysis*-fed group, the copepod enriched-fed group and the shrimp post larvae-fed group in terms of wet weight, total length and gamete diameter were determined and calculated using one-way ANOVA (IBM SPSS Statistics 24) with the Tukey's *post hoc* test (p -value = 0.05)

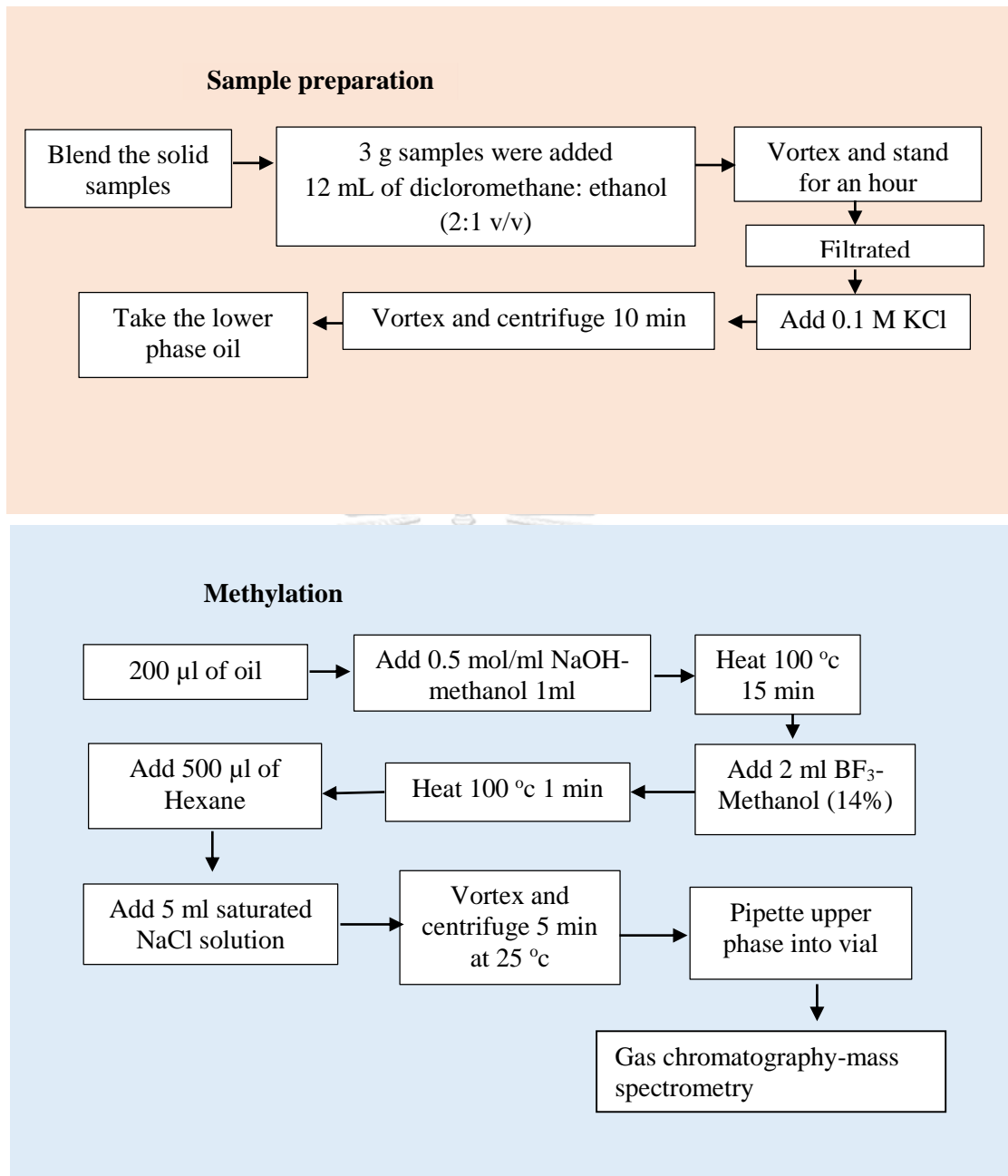


Figure 3-3 The fatty acid analyzed in the feeding treatments and the body fatty acid of seahorse.

CHAPTER 4

RESULTS

Growth and Survival of Hippocampus sp. During Juvenile Stages

The survival rates of the *Hippocampus* sp. juveniles were estimated in two different periods: 10 days after birth (DAB) and 31 DAB. The first mortality rate of the juveniles was observed at the 5 DAB in the *Artemia*-fed group. At 10 DAB, the lowest survival rate was observed in the *Artemia*-fed group as 90% of the juveniles were died. At 10 DAB, all of the juveniles fed with the *Artemia* were observed all mortality, while the highest survival rate was observed in the *Mysis*-fed group at 86% and followed by the enriched-copepod fed group of 77% and the post larvae shrimp-fed group of 57%, respectively. At 31 DAB of the study, the survival rates of the *Mysis*-fed group were still at the highest at 86% which was higher than the enriched copepod-fed group of 70% and the post-larvae shrimp-fed group of 57%, respectively as displayed in Fig 4-1.

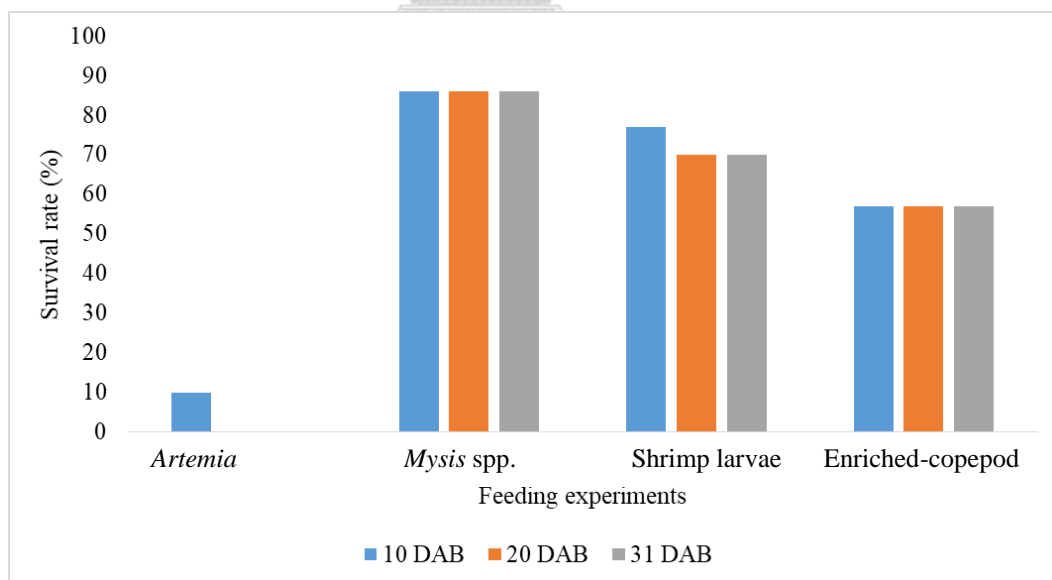


Figure 4-1 Survival rates (%) of *Hippocampus* sp. at 10 DAB, 20 DAB, and 31 DAB in the *Artemia*-fed group, the *Mysis*-fed group, the enriched copepod-fed group, and the post larvae shrimp-fed group. DAB day after birth; (n=32)

The growth rates of the juveniles *Hippocampus* sp. were estimated into 3 different periods including 10 DAB, 20 DAB and 31 DAB. Total length (TL, mean \pm SE, $n=3$) at 10 DAB of the *Mysis*-fed group (35.33 ± 1.76 mm) and the post larvae shrimp-fed group (30.07 ± 0.56 mm) were observed as the highest TL. There were no significant differences between the length (mean \pm SE) of the *Artemia*-fed treatment (23.23 ± 0.12 mm) and the enriched copepods-fed group (26.87 ± 2 mm) ($p>0.05$), which were observed as the lowest TL. At 20 DAB of the study, the *Mysis*-fed group (40.5 ± 3.77 mm) was observed as the highest TL while the post larvae shrimp-fed group (35.2 ± 1.9 mm) was observed the lowest TL. At 31 DAB of the study, there were no significant differences between the TL (mean \pm SE) of the *Mysis*-fed group (57.3 ± 3.75 mm) and the post larvae shrimp-fed group (52.8 ± 2.62 mm) ($p>0.05$), which were observed the highest TL while the enriched copepods-fed group (41.87 ± 1.36 mm) was observed the lowest TL (Fig 4-2, 4-6) and (Table 4-1).

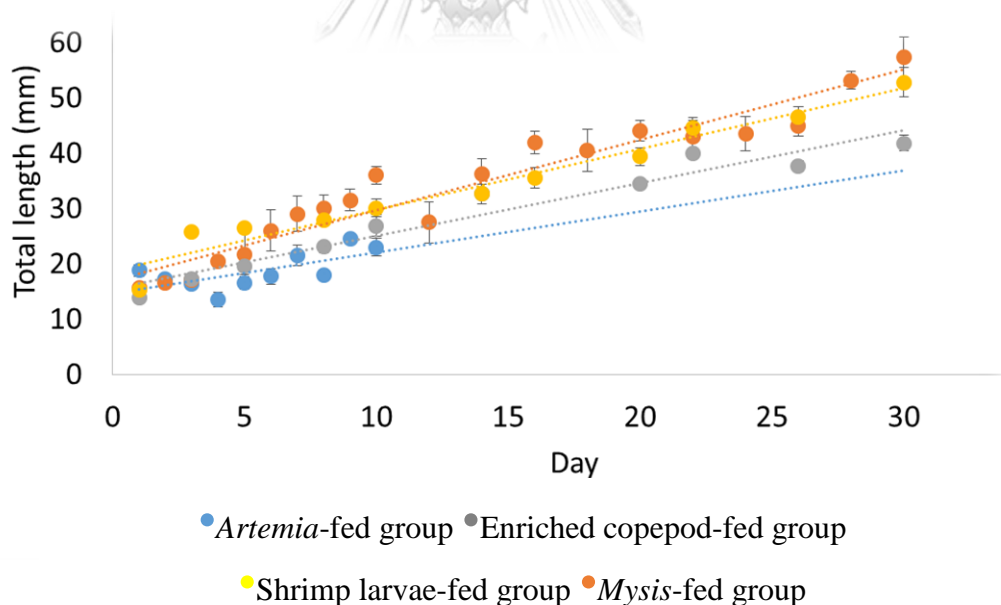


Figure 4-2 The total length (mm) of *Hippocampus* sp. sine the day of birth to the 31 DAB in the *Artemia*-fed group, the *Mysis*-fed group, the enriched copepod-fed group, and the post larvae shrimp-fed group; DAB day after birth; ($n=3$).

The wet weights (WW; mean \pm SE, $n=3$) of the *Hippocampus* sp. at 10 DAB were significant difference between the feeding groups ($p<0.05$), the *Mysis*-fed group (74.38 ± 5.04 mg) had higher than the post larvae shrimp-fed group (62.78 ± 6.29 mg), the enriched copepods-fed group (46.87 ± 2.57 mg), and the *Artemia*-fed group (8.78 ± 0.56 mg), respectively. At 20 DAB, all of the feeding group had no difference in WW. At 31 DAB, the *Mysis*-fed group was observed the highest weight (443.3 ± 18.86 mg) which followed by the post larvae shrimp-fed group (333.7 ± 9.04 mg) and the enriched copepods-fed group (123.39 ± 4.74 mg), respectively (Fig 4-3) and (Table 4-1).

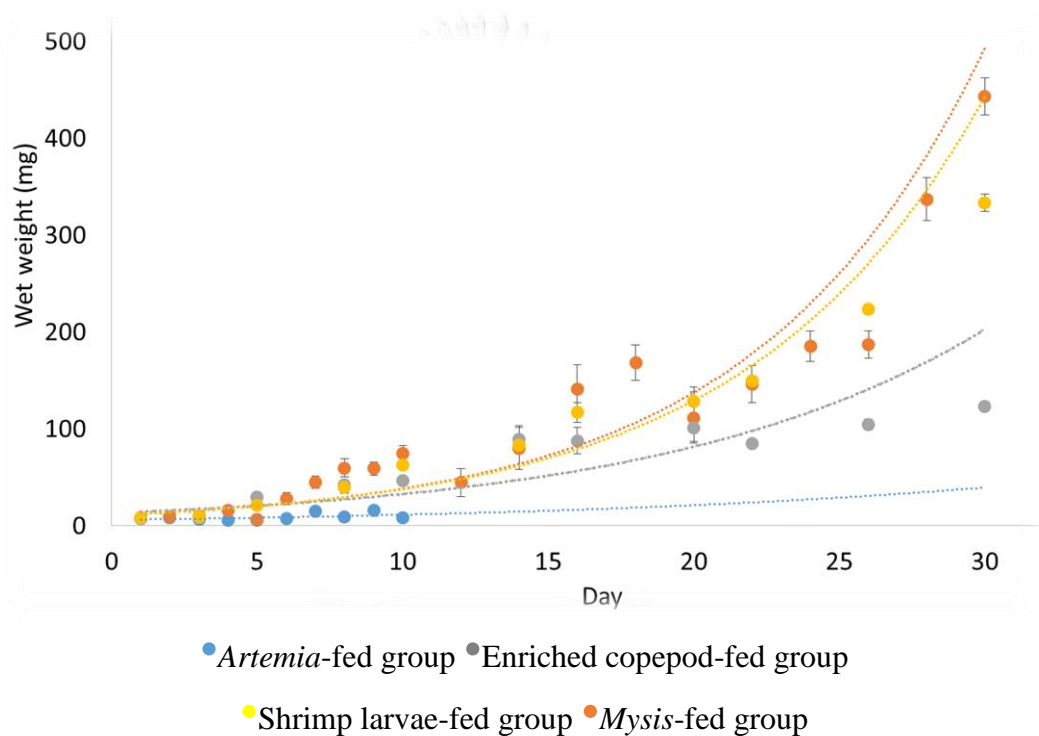


Figure 4-3 The wet weight (mg) of *Hippocampus* sp. at the day of birth to the 31 DAB in the *Artemia*-fed group, the *Mysis*-fed group, the enriched copepod-fed group, and the post larvae shrimp-fed group; DAB day after birth; ($n=3$).

Condition factors (CF) were estimated at the end of the study. The *Mysis*-fed group showed the highest value in CF (773.65) followed by the post larvae shrimp-fed group (632) and the enriched copepods-fed group (294.69), respectively (Fig 4-4). In the case of the percentage specific growth rate (%SGR), the *Mysis*-fed group showed the highest value (13.2%/day) which followed by the post larvae shrimp-fed group (12.16%/day), the enriched copepods-fed group (9.19%/day) and the *Artemia*-fed group (1.03%/day), respectively (Fig 4-5) and (Table 4-1).

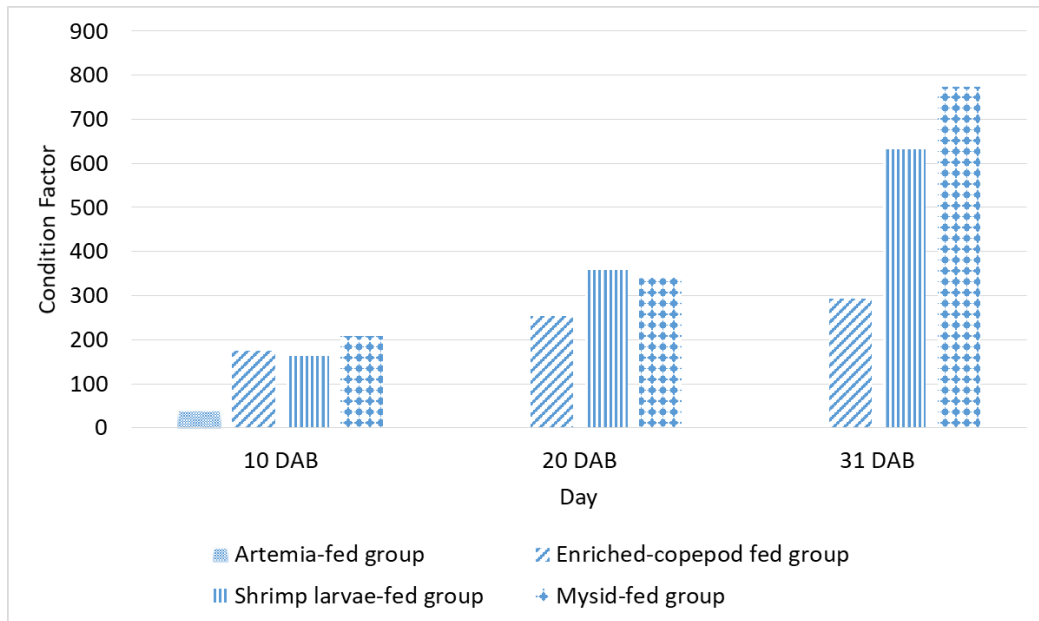


Figure 4-4 The condition factor of *Hippocampus* sp. at 10 DAB, 20 DAB and 31 DAB in the *Artemia*-fed group, the *Mysis*-fed group, the enriched copepod-fed group, and the post larvae shrimp-fed group; DAB day after birth; ($n=3$).

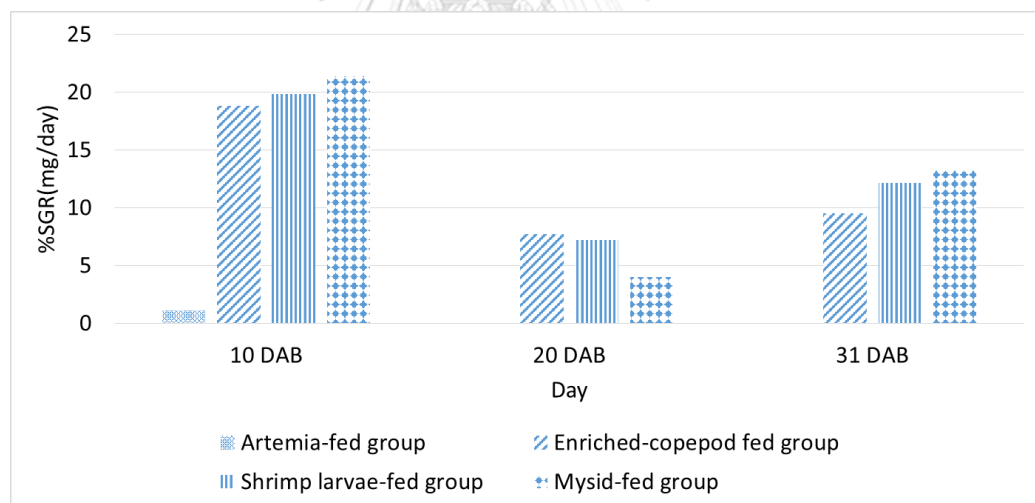


Figure 4-5 The specific growth rate (%/day) of *Hippocampus* sp. at the 10 DAB, 20 DAB and 31 DAB in the *Artemia*-fed group, the *Mysis*-fed group, the enriched copepod-fed group, and the post larvae shrimp-fed group; DAB day after birth; ($n=3$).

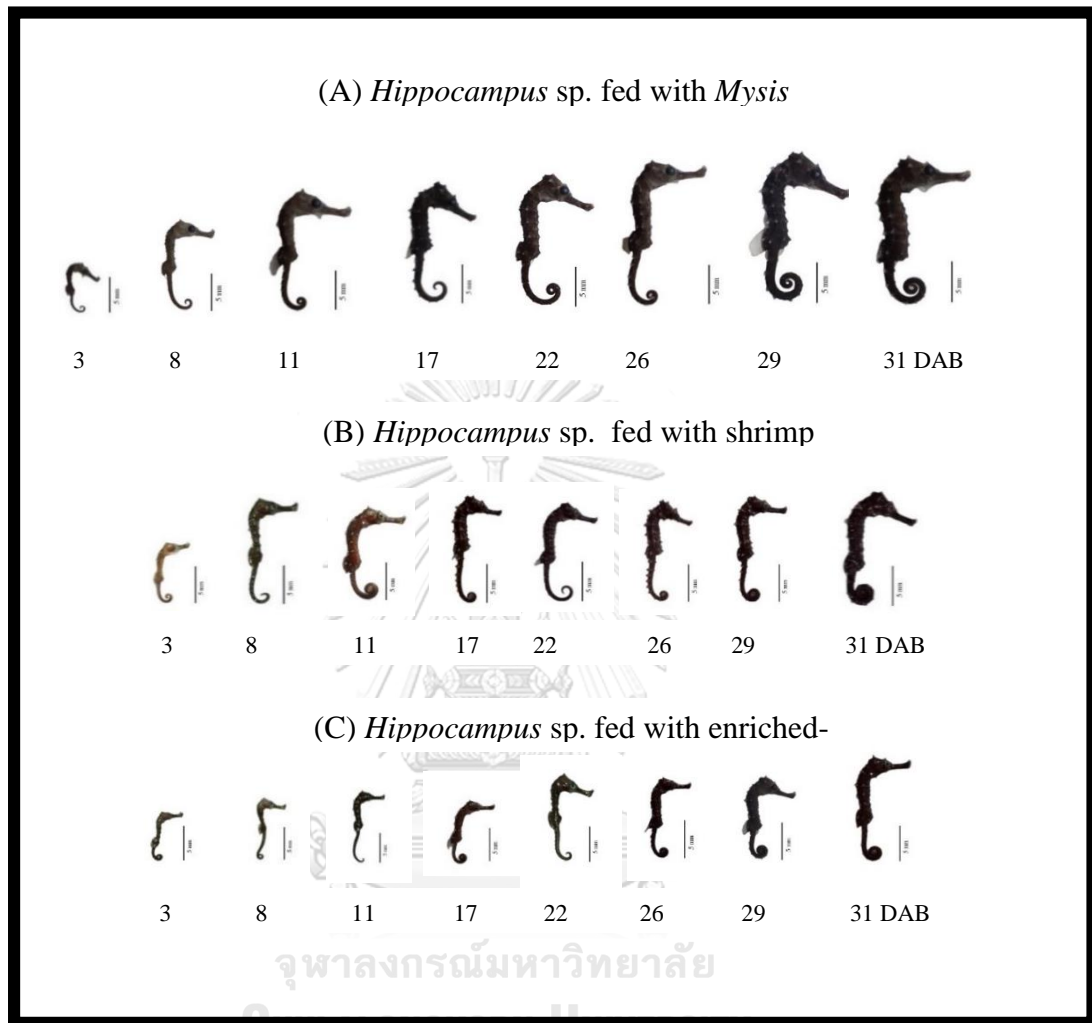


Figure 4-6 *Hippocampus* sp. growth in the feeding experiment (A) *Mysis* spp., (B) shrimp larvae and (C) enriched copepod during day of birth to 31 DAB.

Table 4-1 *Hippocampus* sp. specific growth rate (%/day), condition factor, wet weight (mg), and total length (mm) in 10 DAB, 20 DAB, and 31 DAB. The different superscripts in the same column show significant differences ($P < 0.05$) within the same day in feeding treatment; DAB day after birth.

Experimental data	<u>Artemia</u> -fed seahorse			Mysis- fed seahorse			Enriched-Copepods- fed seahorse			Post larvae shrimp- fed seahorse		
	10 DAB	20 DAB	30 DAB	10 DAB	20 DAB	30 DAB	10 DAB	20 DAB	30 DAB	10 DAB	20 DAB	30 DAB
Specific growth rate (%/day)	1.1	-	-	21.4	4	13.2	18.8	7.7	9.49	19.8	7.2	12.16
Condition factor	38.3	-	-	208.69	341.1	773.65	174.43	253.99	294.69	164.91	358.80	632
Wet weight (mg)	8.78 ±0.56 ^a	-	-	74.38 ±5.04 ^b	111.79 ±26.37 ^a	443.3 ±18.86 ^a	46.87 ±2.57 ^c	101.52 ±14.22 ^a	123.39 ±4.74 ^b	62.78 ±6.29 ^d	128.09 ±10.14 ^a	333.7 ±9.04 ^c
Total length (mm)	23.23 ±0.12 ^a	-	-	35.33 ±1.76 ^b	40.5 ±3.77 ^a	57.3 ±3.75 ^a	26.87 ±2 ^{ac}	39.97 ±0.95 ^a	41.87 ±1.36 ^b	30.07 ±0.56 ^b	35.2 ±1.9 ^b	52.8 ±2.62 ^a

Female reproductive system of Hippocampus sp.

Gonadal differentiated ratio of Hippocampus sp.

The gonadal structure and differentiation in the *Hippocampus* sp. samples from the day of birth until the 31 DAB were determined using histological procedures. The *Hippocampus* sp. juveniles were observed in ovary and undifferentiated gonad throughout the study. The ratio of the ovary and the undifferentiated gonad were 1.52 : 1.

An Overview of Gonadal Structure and Gametogenesis of Hippocampus sp.

The female reproductive system of *Hippocampus* sp. consisted of ovary with the ovarian lumen. The symmetrical ovary was a paired and elongated cylindrical organ (Fig 4-7A). Under histological section, ovary lied posteriorly in the coelomic cavity. The ovary located between a kidney and posterior intestine (Fig 4-7A) and was suspended by the mesovarium (Fig 4-7B).

The ovary of *Hippocampus* sp. was enclosed by a thin layer of tunica albuginea (or the ovarian capsule) (Fig 4-8A). A few muscular layer and some blood vessels in most of connective tissues were observed in tunica albigena (Fig 4-8A). Within the ovarian tissue, different stages of oocytes were observed which this feature was considered to be an asynchronous developmental oocytes (Fig 4-7B). The ovarian parenchyma of *Hippocampus* sp. could be divided into 2 compartments: germinal compartment and stromal compartment. The germinal compartment composed of a single layer of germinal epithelium and oogonial cysts (Fig 4-8B) which oogonium (OG) was about 3-7 μm in diameter was visible in the ovarian cysts. OG exhibited an ovoid shape with the deep blue stained of heterochromatin in nucleus. Oocyte was also surrounded by pre-follicular cell. During differentiation process, OG continuously grow into oocyte in the stromal compartment. In stromal compartment, histological observation revealed the developing oocytes which could divided into 2 steps into primary growth stage and secondary growth stage. The developing oocyte were classified based on size, histological stain, cell features, and folliculogenesis, which details were given in the following subtopics.

Primary Growth Stage (PGs)

PGs were classified into 4 sub-stages including chromatin nucleolus stage (CN), multiple nucleoli stage (MN), perinucleolar stage (PN) and oil droplets and cortical alveoli stage (OC). As to detail, ovoid shape of CN are undergoing 1st meiosis. Nucleus showed centrally placed nucleolus. CN attained in a diameter of 9-15 μm . This stage was also surrounded by a pale basophilic cytoplasm. MN was attained of the 9-35 μm diameter and multiple nucleoli were distributed in the nucleus. Ooplasm of MN was intensely basophilic stained, when compared with the previous stage. PN was still ovoid shape and increased in size of 26-50 μm . Nucleoli of PN were clearly arranged in periphery of nucleus which ooplasm exhibited intense stained of eosin. OC was characterized by an increasing in cell size of 70-120 μm compared with the previous stages of oocyte. OC decreased in nucleo-cytoplasmic ratio and cytoplasm was initially accumulated in two inclusions (the lipid droplet and cortical alveoli). The spherical lipid droplets were seen as empty due to their lipid dissolves during histological technique. The localization of this inclusion was detected in close to the follicular complex. The oval cortical alveoli were observed with clear content vesicle. A well-development of the follicular complex was consisted of zona pellucida and a single layer of follicular cell were formed, the oocyte differentiation were showed in Fig 4-7B.

Secondary Growth Stage (SGs)

SGs was characterized by substantial increasing in 100-120 μm diameter when compared with OC stage. Strength of the pinkness staining in ooplasm was increased, as positive reaction with PAS method, and ooplasm initial contained in numerous small yolk granules. Lipid droplets and cortical alveoli was still seen, but increase in size and abundant throughout the ooplasm. A well development of the zona pellucida and a simple layer of follicular cells was observed in this stage (Fig 4-9B).

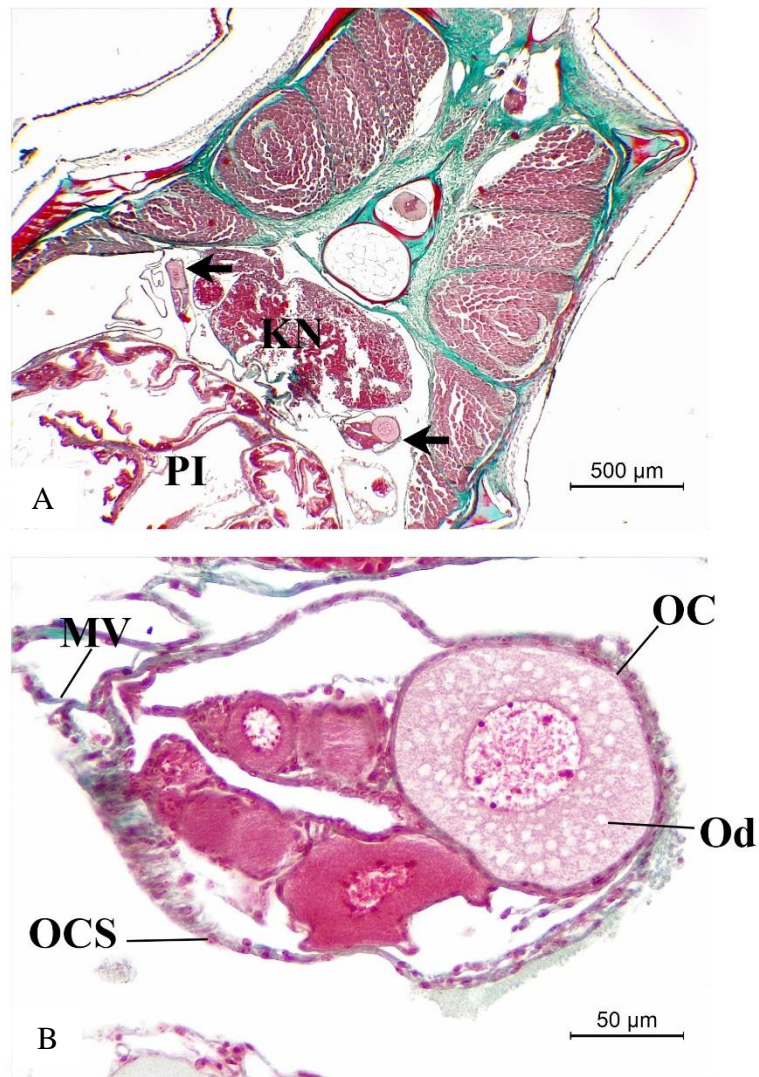


Figure 4-7 (A) and (B) The *Hippocampus* sp. ovary (arrow) with the developing oocytes was a paired and elongated cylindrical organ. It was suspended by the mesovarium (MV) between a kidney (KN) and posterior intestine. The ovary was enclosed by the ovarian capsule (OCS); KN kidney, PI posterior intestine, MV mesovarium, OC oil droplets and cortical alveoli stage, OCS ovarian capsule, Od oil droplets; Masson Trichrome stained.

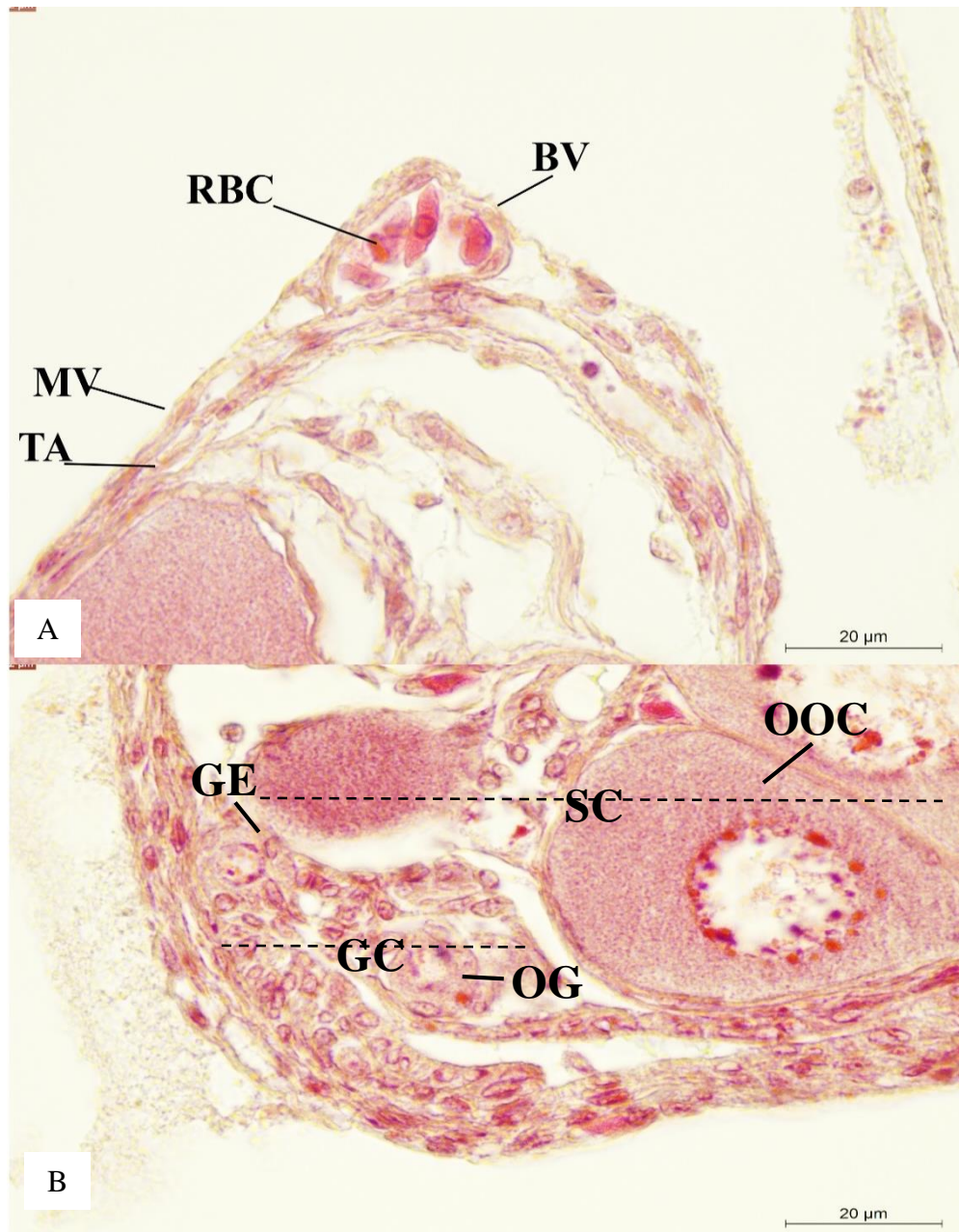


Figure 4-8 (A) The ovary of *Hippocampus* sp. was enclosed by a thin layer of tunica albuginea (TA) and a few muscular wall (MV) with some blood vessels (BV). (B) The ovarian parenchyma of *Hippocampus* sp. could be divided into 2 compartments: germinal compartment (GC) and stromal compartment (SC). The germinal compartment composed of germinal epithelium (GE) and oogonia (OG) which was about 3-7 µm in diameter. The stromal compartment composed of the developing oocyte (OOC); TA tunica albuginea, MV muscular wall, BV blood vessel, GC germinal compartment, SC stromal compartment, GE germinal epithelium, OG oogonia, OOC oocyte; H&E stained.

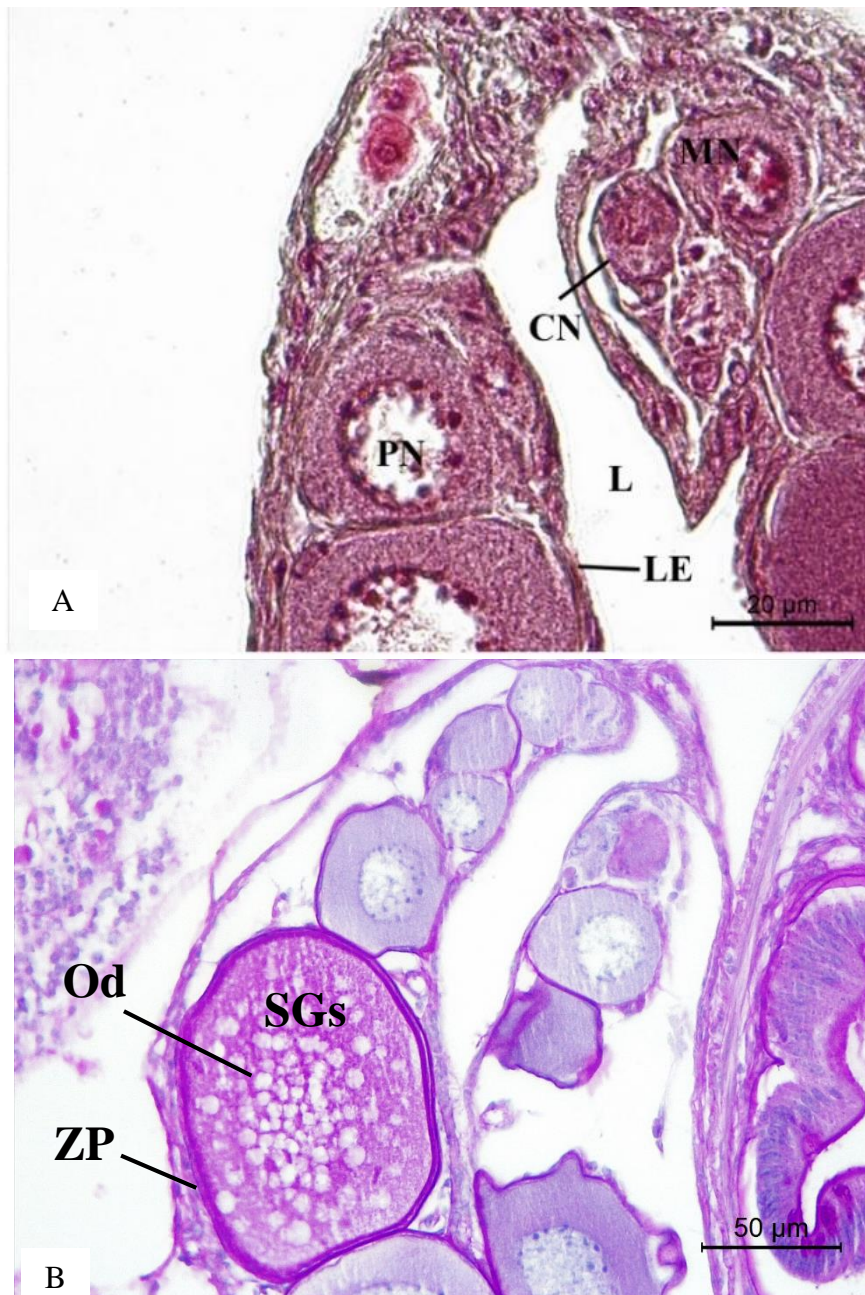


Figure 4-9 (A) and (B): The oocytes in developing primary growth stage including chromatin nucleolus stage (*CN*), multi-nucleolus stage (*MN*), perinucleolus stage (*PN*) and oil droplets and cortical alveoli stage (*OC*) in secondary growth stage (*SGs*) with a well-developed zona pellucida (*ZP*); *CN* chromatin nucleolus stage, *MN* multi-nucleolus stage, *PN* perinucleolus stage, *SGs* secondary growth stage, *Od* oil droplets, *L* lumen, *ZP* zona pellucida; A: H&E stained, B: PAS stained.

Gonadal differentiation of the Hippocampus sp. Related to Diet

In the present study, the effects of the live preys on the gonadal differentiation in *Hippocampus* sp. was divided into three experiments based on differently diets: *Mysis* spp., shrimp larvae, and enriched copepod treatment.

Ovarian Differentiation of the Mysis-Fed Group

In *Hippocampus* sp. fed with *Mysis* spp., the gonadal structure was observed as a small ovoid organ and ventrally located in the kidney. The undifferentiated gonad was observed during 11 day after birth (DAB) and the putative ovarian structure was first observed in 11 DAB. The ovary of the seahorse during 11 to 26 DAB exhibited five principle cells of PGs: OG, CN, MN, PN and a few OC. The ovary showed a few OG ranging from 4-7 μm in diameter, forming in oogonial cysts. Oogonia increased in size and differentiated to oocytes in PGs. CN attained in a diameter of 9-14 μm . MN attained in a diameter of 24-36 μm . This oocyte was distinguished by the multiple nucleoli which were random distributed in the germinal vesicle. PN was distinguished by multiple nucleoli that become located around the periphery of the nucleus where the oocyte were 35-52 μm diameter. At 26 DAB, the ovary of *Hippocampus* sp. appeared OC. The presence of SGc was existed at 31 DAB. OC were exhibited the of 90-110 μm diameter with the accumulated yolk vesicle which has pale pinkness stained of Masson Trichrome (Fig 4-10).

Ovarian Differentiation of the Shrimp Larvae-Fed Group

The ovary of *Hippocampus* sp. fed with the shrimp larvae was observed at 11 day after birth (DAB). Both oogonia and primary growth stage of oocyte were detected which revealed similar histological characteristics as mentioned in the previous group. During 11 DAB and 26 DAB, OG, MN and PN exhibited the same cell-feature compared with the previous feeding group. OG, CN, MN and PN were 4-7 μm , 10-15 μm , 24-43 μm and 30-48 μm in diameter, respectively. At 31 DAB, OC firstly observed attaining a diameter of 48-51 μm . However, SGs were not visible in the experimental group (Fig 4-11).

Ovarian Differentiation of the Enriched Copepod-Fed Group

The ovary of *Hippocampus* sp. fed with the enriched copepod was observed at 17 day after birth (DAB). During the 17–31 DAB, they were observed the oogonia and the oocyte in primary growth. Sizes of OG, CN, MN and PN was about 3-5 μm , 10-15 μm , 9-29 μm and 26-46 μm . Oogonia and PGs fed exhibited the same cell feature compared with the previous feeding group. Nevertheless, at 31 DAB, oocyte still developed in perinucleolus stage of PGs while OC did not appear (Fig 4-12).

Fatty Acid and Crude Protein Analysis

The fatty acid profiles were observed in dietary including the *Mysis* spp., the shrimp larvae and the enriched copepod. Highly unsaturated fatty acid especial in 20:4n-6 (ARA, arachidonic acid), 20:5n-3 (EPA, eicosapentaenoic acid), and 20:6n-3 (DHA, *docosahexaenoic acid*) showed high contents in *Mysis* spp. shrimp larvae and copepods, respectively. Moreover, there were also high contents of fatty acid in 16:0 (palmitic acid), 18:1n-9 (oleic acid) and 18:2n-6 (linoleic acid). The fatty acid profiles of *Hippocampus* sp. showed the similar trend among the feeding treatments, which had a higher percentage composition of 20:4n-6 (ARA), 20:5n-3 (EPA), 20:6n-3 (DHA) 16:0 (palmitic acid), 18:1n-9 (oleic acid) and 18:2n-6 (linoleic acid) in *Mysis* spp. shrimp larvae and copepods, respectively (Fig 4-13 and 4-14).

Total protein content in dietary *Mysis* spp., shrimp larvae, enriched copepod, and *Artemia* were 5.32 g, 5.15 g, 3.89 g, and 4.11 g per 100 g of total weight, respectively.

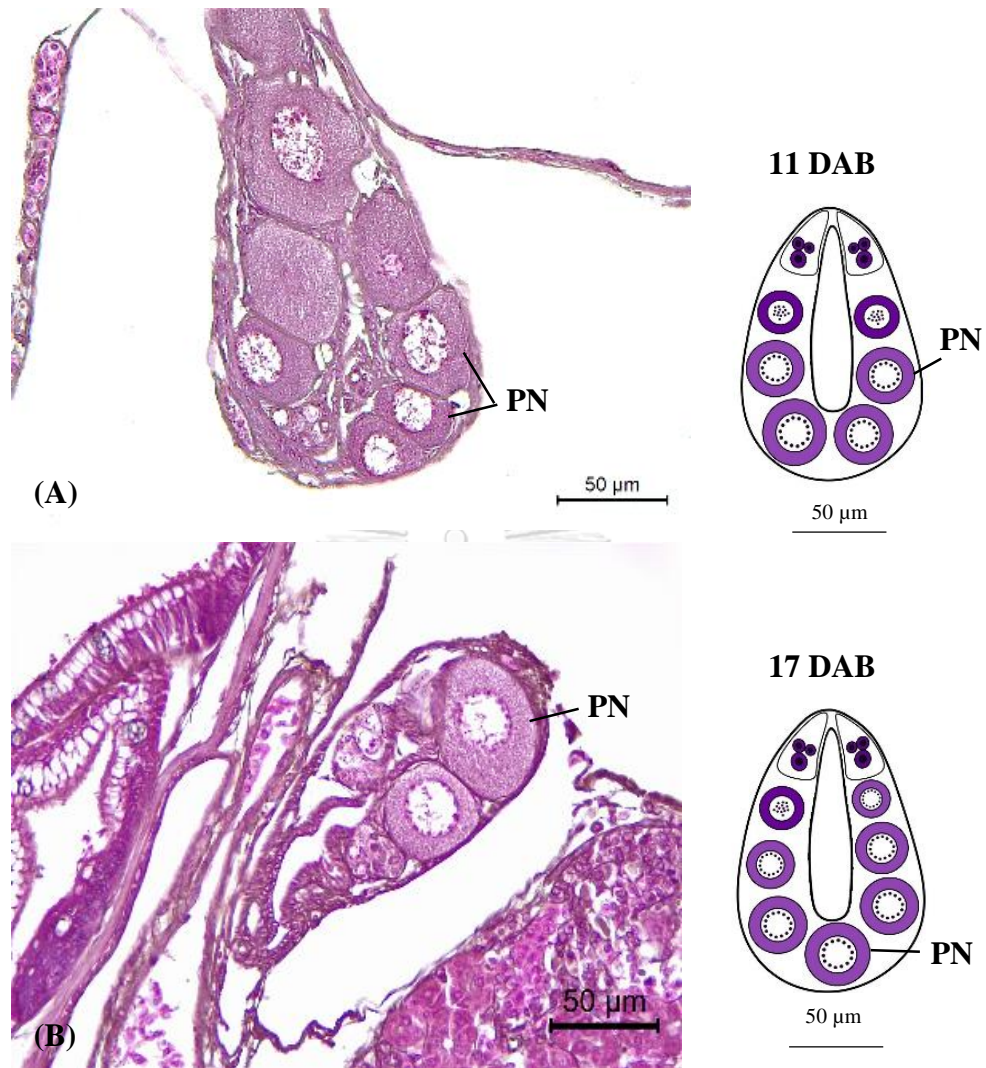


Figure 4-10 Histological feature of *Hippocampus* sp. ovary of *Mysis*-fed group in 11 DAB (A) and 17 DAB (B) were often observed PN; DAB day after birth, PN perinucleolus stage.

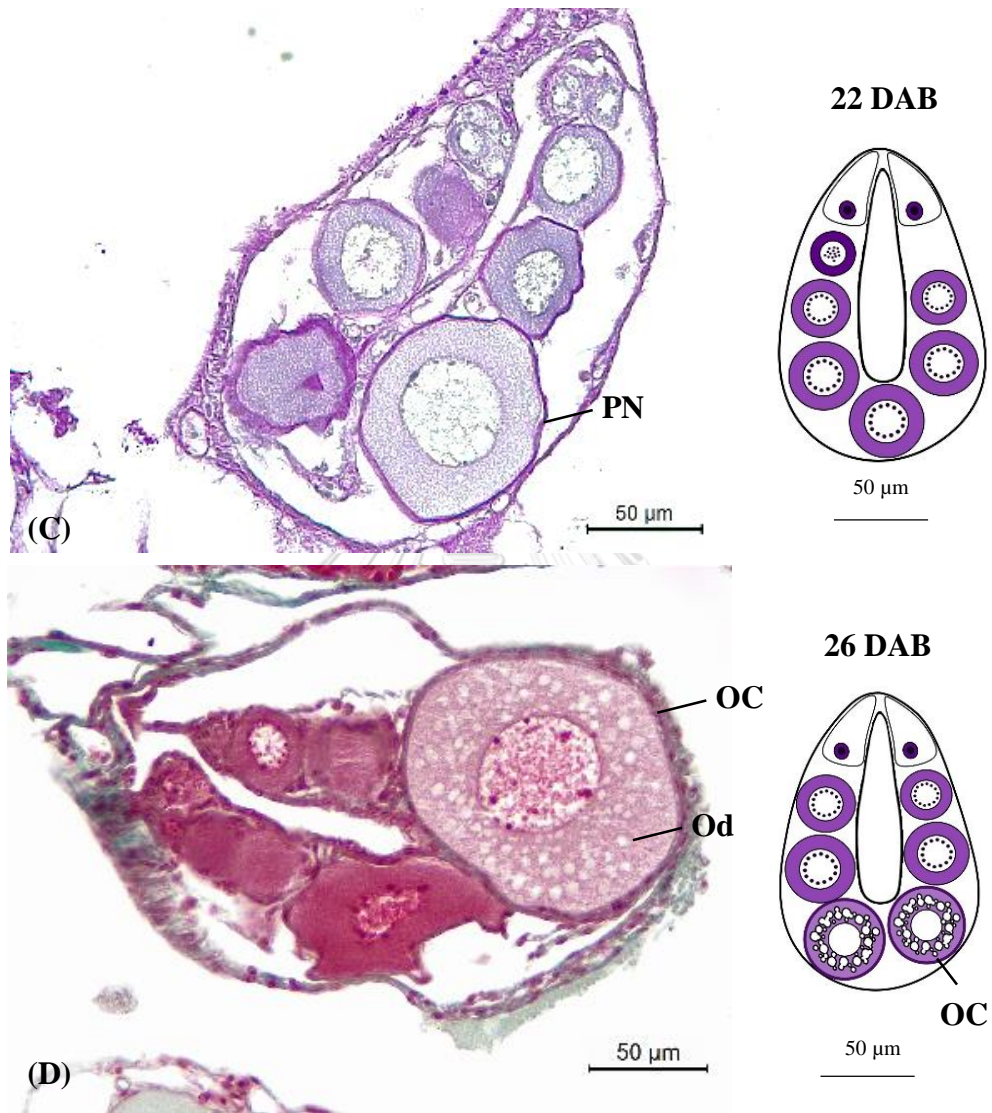


Figure 4-10 (continued) Histological feature of *Hippocampus* sp. ovary of *Mysis*-fed group in 22 DAB (C) was observed PN while in 26 DAB (D) was observed OC which the oil droplets (Od) accumulated in the ooplasm; DAB day after birth, PN perinucleolus stage, OC oil droplets and cortical alveoli, Od oil droplets.

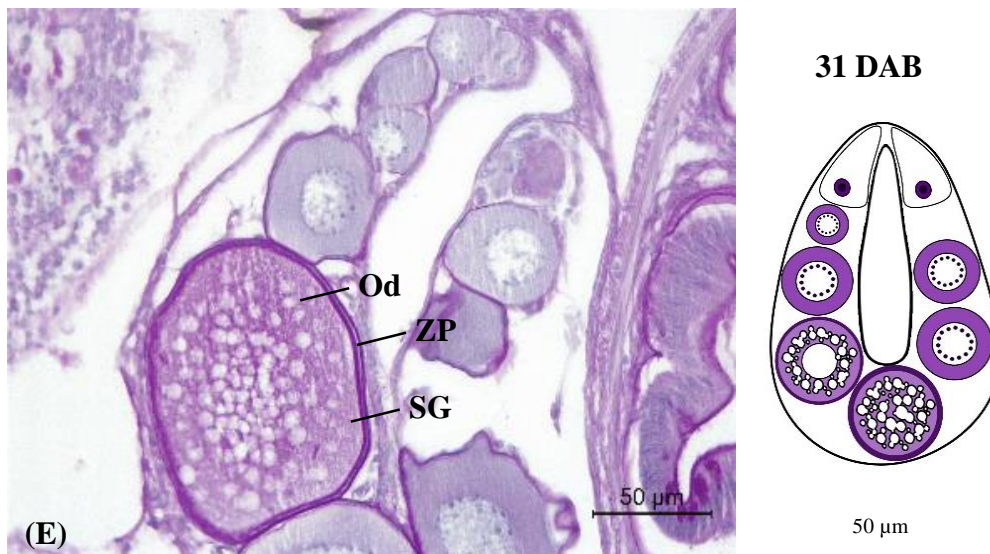


Figure 4-10 (continued) Histological feature of *Hippocampus* sp. ovary of *Mysis*-fed group in 31 DAB (E) was observed SGs which the dispersed oil droplets increase in size in ooplasm. SG was absent germinal vesicle and it showed the thickness in zona pellucida (ZP).; DAB day after birth, SG secondary growth stage, Od oil droplets, ZP zona pellucida.

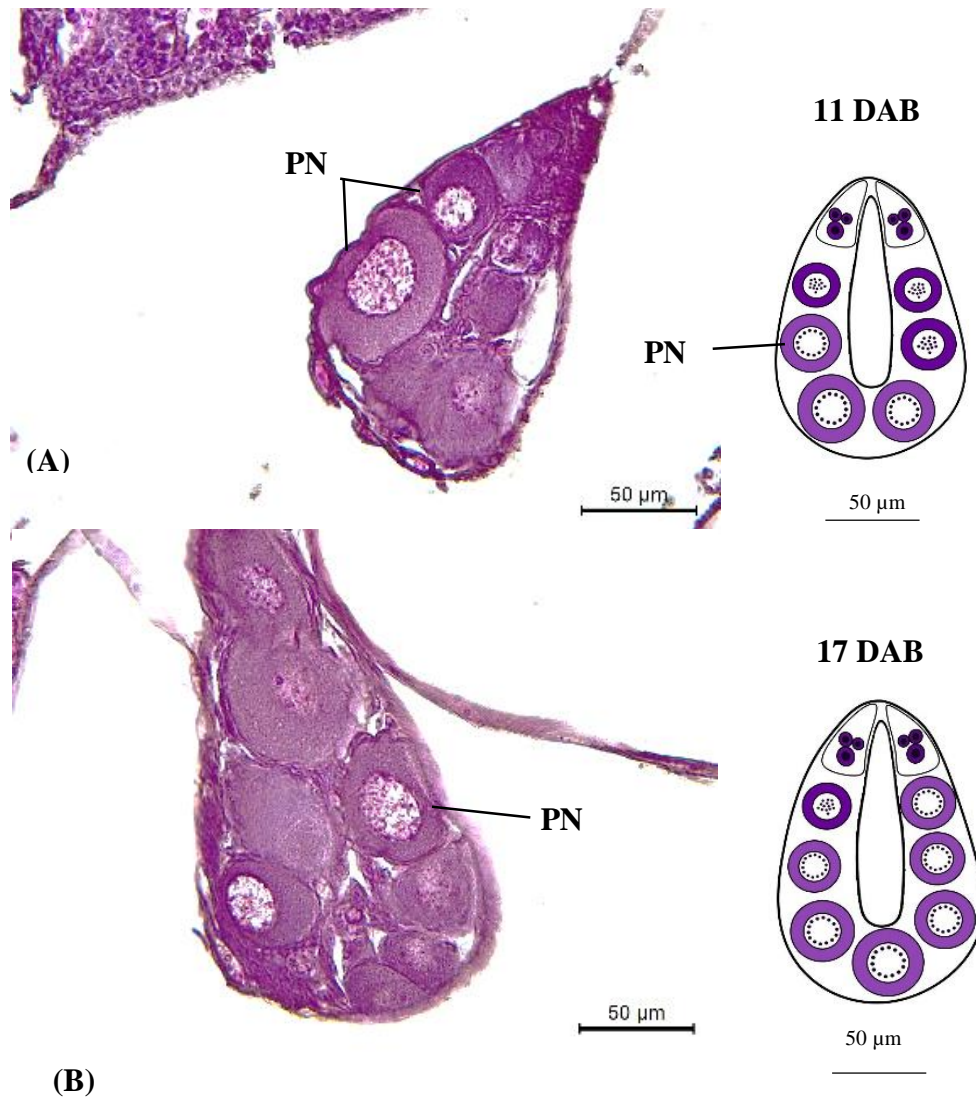


Figure 4-11 Histological feature of *Hippocampus* sp. ovary of shrimp larvae-fed group in 11 DAB (A) and 17 DAB (B) were often observed PN; DAB day after birth, PN perinucleolus stage.

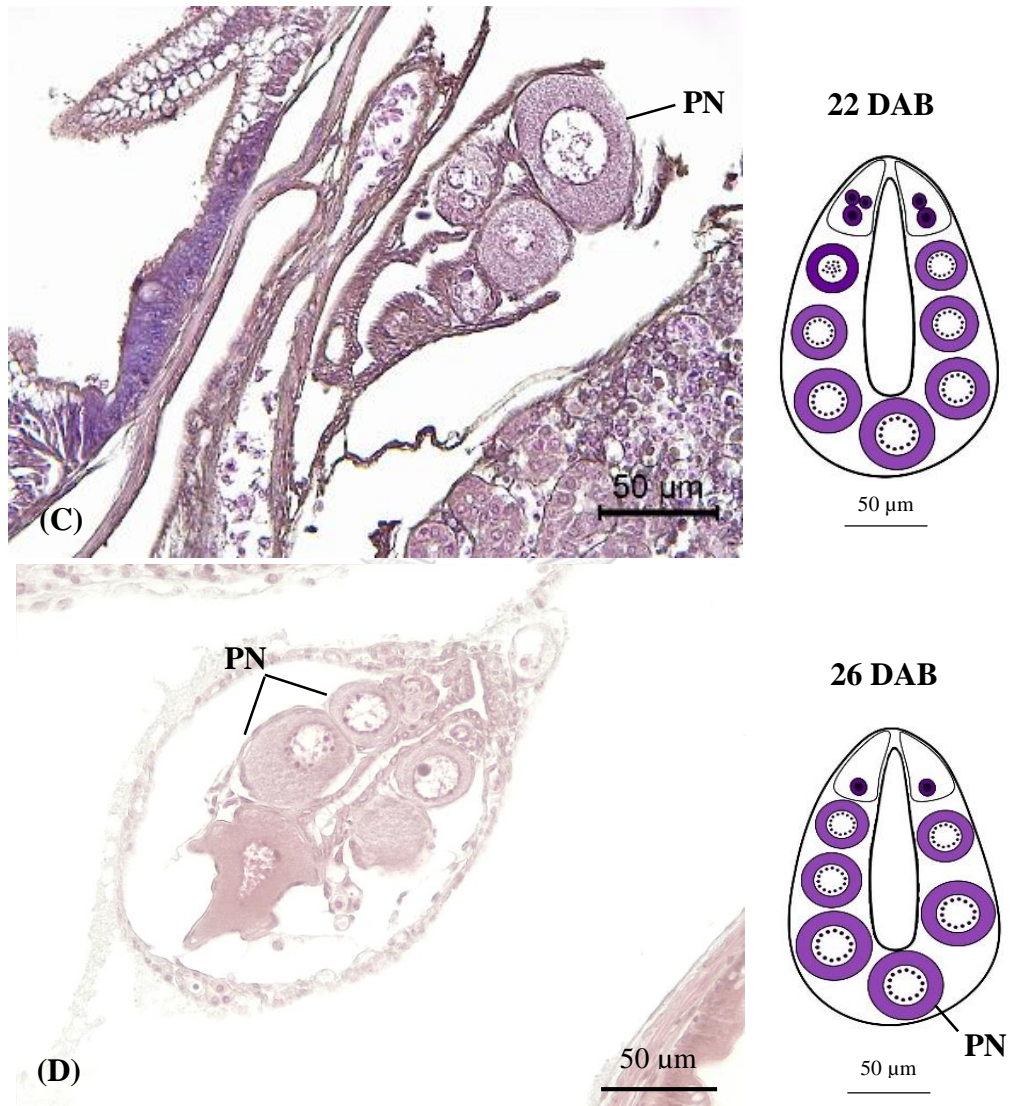


Figure 4-11 (continued) Histological feature of *Hippocampus* sp. ovary of shrimp larvae-fed group in 22 DAB (C) and 26 DAB (D) were observed PN; DAB day after birth, PN perinucleolus stage.

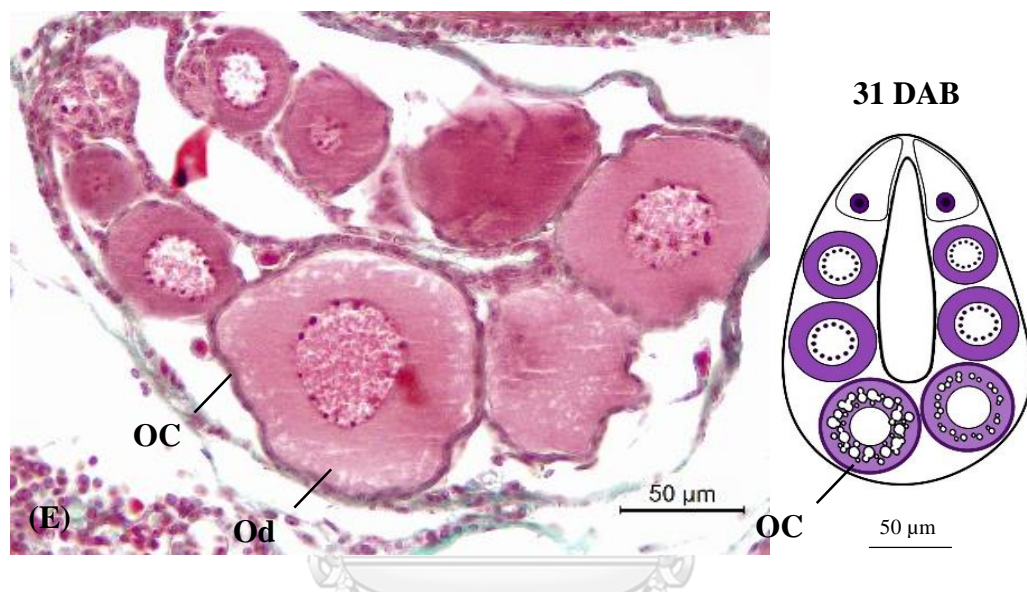


Figure 4-11 (continued) Histological feature of *Hippocampus* sp. ovary of shrimp larvae-fed group in 31 DAB was observed OC which the oil droplets (*Od*) accumulated periphery in the ooplasm; *DAB* day after birth, *OC* oil droplets and cortical alveoli, *Od* oil droplets.

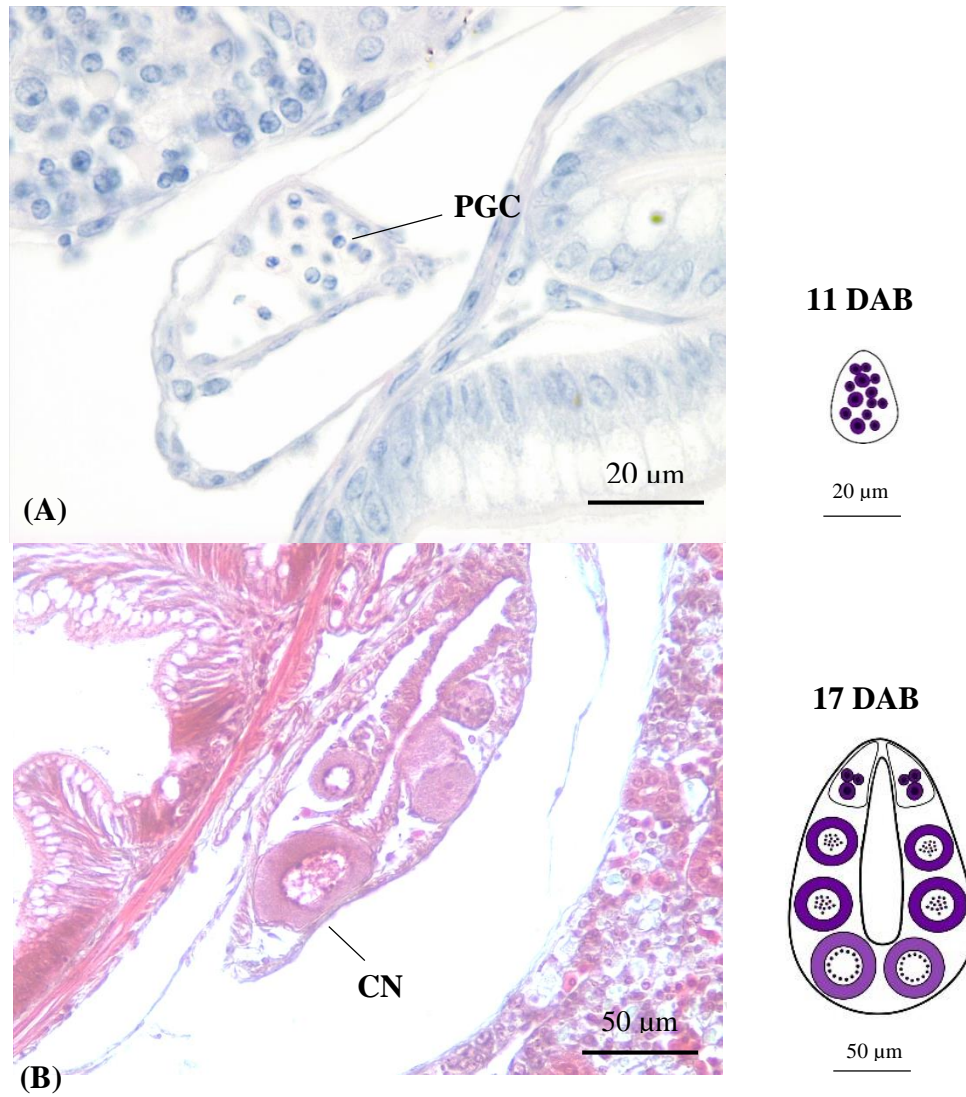


Figure 4-12 Histological feature of *Hippocampus* sp. primordial gonad of enriched copepod-fed group in 11 DAB (A) was observed the primordial germ cell in the gonad. At 17 DAB (B), the ovary was observed PN; DAB day after birth, PGC primordial germ cell, PN perinucleolus stage.

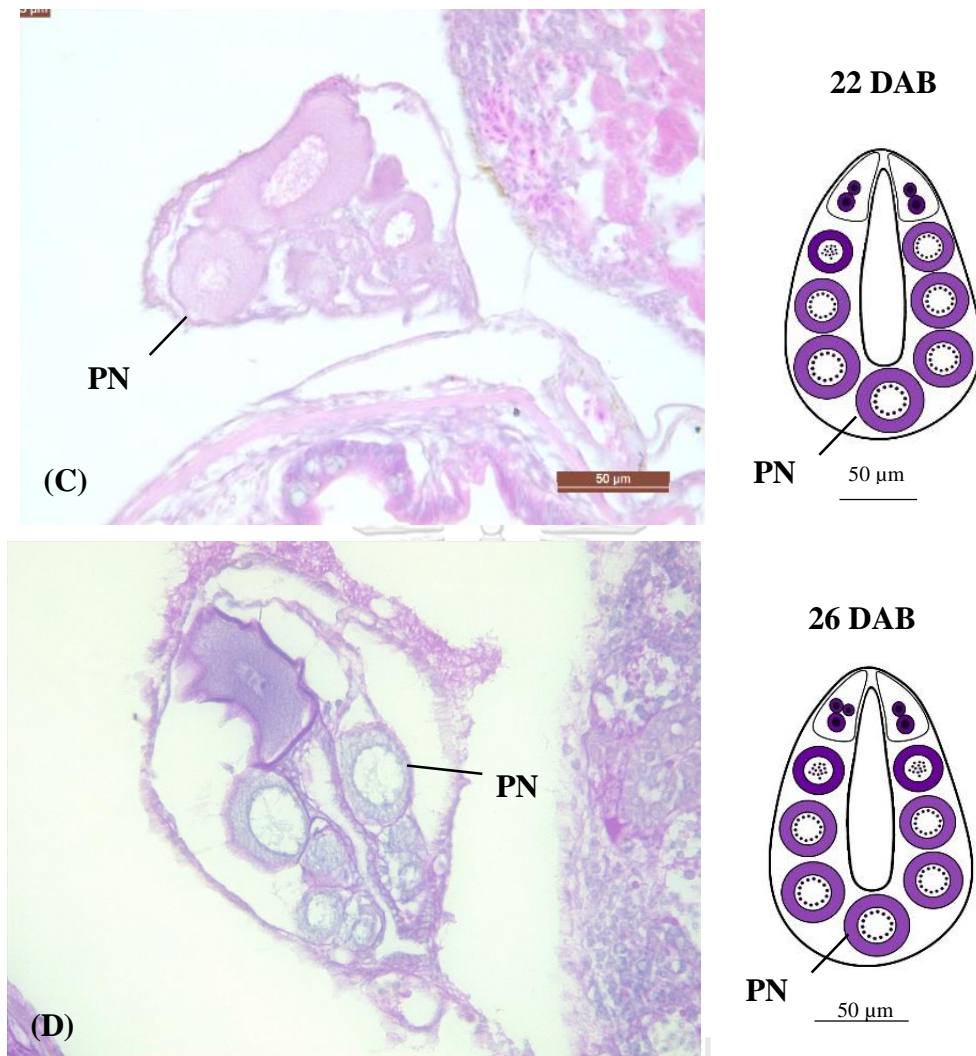


Figure 4-12 (continued) Histological feature of *Hippocampus* sp. ovary of enriched copepod-fed group in 22 DAB (C) and 26 DAB (D) were observed PN; DAB day after birth, PN perinucleolus stage.

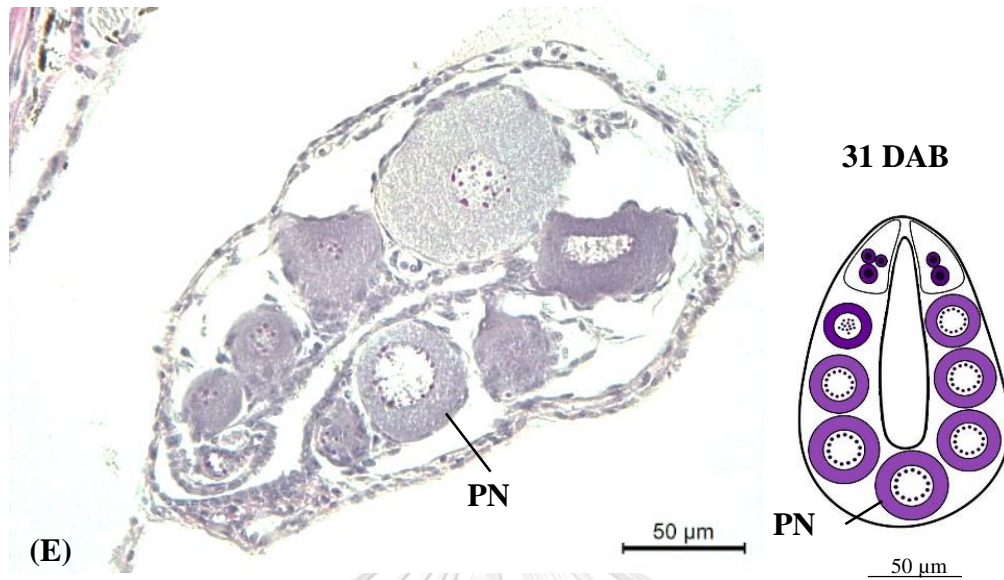


Figure 4-12 (continued) Histological feature of *Hippocampus* sp. ovary of enriched copepod-fed group in 31 DAB was observed PN; DAB day after birth, PN perinucleolus stage.

Table 4-2 Oocyte diameter (μm) in PGs and SGs sub-divided into (A) Chromatin nucleolus stage, (B) Multi-nucleolus stage, (C) Perinucleolus stage, (D) Oil droplets and cortical alveoli stage and (E) early SGs oocyte of *Hippocampus* sp. at 11 to 31 DAB , (-) unavailable data; The different superscripts show different in the experimental treatment ($p < 0.05$).

(A) Chromatin nucleolus stage (CN) of PGs

Day	Feeding treatments		
	<i>Mysis</i> spp.	Shrimp larvae	Enriched copepod
11	11.6 \pm 0.82	13.3 \pm 0.95	-
17	12.1 \pm 1.52	12.3 \pm 1.5	11 \pm 1
22	10.7 \pm 1.4	11.6 \pm 0.58	11.2 \pm 0.7
26	10.2 \pm 0.58 ^a	11.3 \pm 0.57 ^{ab}	12.5 \pm 0.7 ^b
31	11.6 \pm 0.9	12.6 \pm 1.52	13.5 \pm 0.7

(B) Multi-nucleolus stage (MN) of PGs

Day	Feeding treatments		
	<i>Mysis</i> spp.	Shrimp larvae	Enriched copepod
11	29.33 \pm 4.5	35.3 \pm 5.1	-
17	35.6 \pm 11.9 ^a	33.3 \pm 9.1 ^a	14.6 \pm 4.7 ^b
22	36.5 \pm 6.3 ^a	34.6 \pm 3.2 ^a	19 \pm 2.4 ^b
26	37.5 \pm 4.9 ^a	36 \pm 4.5 ^a	26 \pm 3.4 ^b
31	33.5 \pm 2.1 ^a	33.5 \pm 2.1 ^a	24.5 \pm 4.5 ^b

(C) Perinucleolus stage (PN) of PGs

Day	Feeding treatments		
	<i>Mysis</i> spp.	Shrimp larvae	Enriched copepod
11	41.3±6.5	39±9	-
17	40±6.67 ^a	39.2±2.6 ^a	28.6±2 ^b
22	36.7±5.5 ^a	37±6 ^a	30.6±3 ^b
26	35±4.7	36±4.5	36.6±2
31	40±12.3	39±4	41.6±4.5

(D) Oil droplets and cortical alveoli stage (OC) of PGs

Day	Feeding treatments		
	<i>Mysis</i> spp.	Shrimp larvae	Enriched copepod
11	-	-	-
17	-	-	-
22	-	-	-
26	97.5±17	-	-
31	83.3±9	50±1.4	-

(E) Oil droplets and cortical alveoli stage (OC) of SGs

Day	Feeding treatments		
	<i>Mysis</i> spp.	Shrimp larvae	Enriched copepod
31	103.5±5	-	-

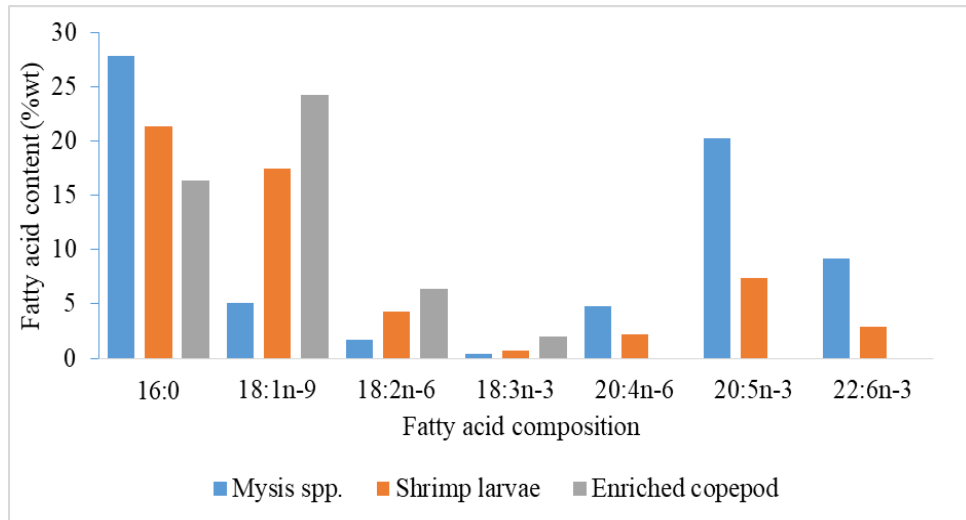


Figure 4-13 The fatty acid content (%) in different feeding treatments: the *Mysis spp.*, the shrimp larvae and the enriched-copepod.

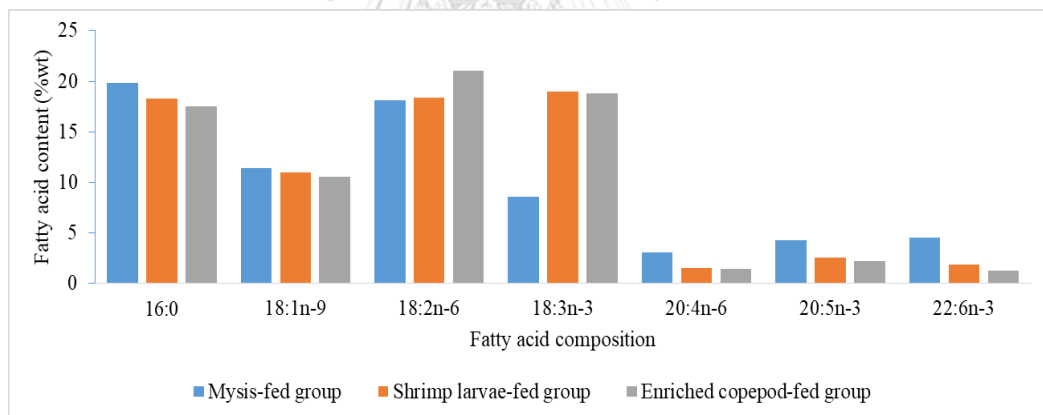


Figure 4-14 The fatty acid content (%) in the *Hippocampus sp.* fed with the *Mysis spp.*, the shrimp larvae and the enriched-copepod.

CHAPTER 5

DISCUSSIONS

Feeding Treatments on Growth Hippocampus sp.

Palma et al. (2014) suggested that the seahorse can consume yolk when they still in the male's pouch. Therefore, the newborns seahorse have to feed after released from brood pouch. Thus, the dietary during the first feeding could influence to newborn seahorse growth and development (Job et al., 2002; Murugan et al., 2009). According to the present study, the experimental groups were conducted under the environmental controlled conditions e.g. salinity, temperature, light. Therefore the newborn *Hippocampus sp.* growth may directly be affected only from the diet composition from the prey.

Referring to the data from this study, *Hippocampus sp.* lived as planktonic larvae during the first 2 weeks after birth which associated with rapid growth during the early life that was represented as SGR of 21.4%. This pattern of growth rate was similar to the results by Novelli et al. (2015) which the newborn *Hippocampus reidi* had the high SGR of 19 %. Within 10 days after birth (DAB) and 31 DAB, they displayed the highest survival rate and growth in the *Mysis*-fed treatment followed by the shrimp larvae fed group, the enriched copepod-fed group and the *Artemia*-fed group. The fatty acid contents in preys could affect the survival rate and growth of the newborn fish. The present results may imply that the wild *Mysis spp.* have the greatest effect on the survival rate and growth of the newborn *Hippocampus sp.* relating to fatty acid composition in the preys. In the case of high specific growth rate (SRG) and survival rate of *Hippocampus sp.* in the *Mysis*-fed group and the shrimp larvae-fed group similar to the previous study in teleosts fish growth. The correlate of the growth with the high content of HUFA in prey revealed in other seahorses, *Hippocampus kuda* (Lin et al., 2007), *Hippocampus whitei* (Wong and Benzie, 2003), *Hippocampus guttulatus* (Palma et al., 2014), *Hippocampus hippocampus* (Otero-Ferrer et al., 2010), *Hippocampus subelongatus* (Payne and Rippingale, 2000) as well as in other marine fish *Paralichthys olivaceus* (Seikai et al., 1997), *Limanda ferruginea* (Copeman et al., 2002).

The mortality of the newborns *Hippocampus* sp. was first observed in the *Artemia* fed group, which 100% mortality occurred at the 11 DAB. This result is similar to the study by Otero-Ferrer et al. (2010) that newborn *Hippocampus hippocampus* fed with the *Artemia* showed high mortality rate. *Artemia*, which particularly used in the larvae culture, have the low contents of HUFA (Sorgeloos et al., 2001; Webster and Lovell, 1990). The high mortality rate may be correlated with some abnormally developed organ. Novelli et al. (2015) revealed that the newborn seahorses were noticed by abnormal swimming behavior and inability of body balance while they ambush the prey. Also, The incomplete maturation of digestive tract before metamorphosis was observed in *Hippocampus reidi* as well as the study of Palma et al. (2014), all of the juvenile *Hippocampus guttulatus* died at 5 DAB due to gas-filled in gas bladder. An inefficient inflation of the swim bladder were also observed in the larvae of other species. This could cause in reduction in growth rates and in mortality rates (Chatain, 1986; Chatain and Dewavrin, 1989). Therefore, the result that mentioned above could imply that the brine shrimp were not appropriate to feed the newborn *Hippocampus* sp.. As they could not provide optimal energy for growth, the survival rate which associated with the less developed in the feeding and swimming organ during larvae stage (Osse et al., 1997).

In particular, the fatty acids with long chain hydrocarbon especially in the highly unsaturated fatty acids (HUFA) play the important role in several aspects e.g. growth performance, immune responses, metabolic energy sources and membrane fluidity. The important HUFAs in marine fish were eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) (Watanabe, 1982; Yone, 1978) while in directly diet arachidonic acid (20:4n-6, ARA) has a few to be occurred. The result in the case of radioactive substrates strongly suggested that the marine species could not produce EPA and ARA (Cowey et al., 1976; Owen et al., 1975). Therefore, the optimal dietary composition may be one of the critical factors that the fish could be obtained only from food intake.

According to the present study as well as the previous reports, the results can imply that HUFAs could affect survival rates and growth performances correlated with coordination of organ systems in fish. The well development and growth of

Hippocampus sp. may be associated with the good health. Essential fatty acids intake could lead to increasing in immune responses and increasing in antibody productions (Kiron et al., 1995). The study by Sheldon Jr and Blazer (1991) showed that an increasing macrophage in kidney was associated with the increased in levels of n-3 fatty acids in dietary which could reduce the mortality rate in the organism. The development of neural and visual functions in marine fish could be affected by obtaining DHA (Bell and Dick, 1991). DHA and EPA play the similar role which can affect fish growth and survival rates with the notice that assimilation of DHA was higher than EPA (Koven et al., 1993). Well develop of neural and visual functions improve the effective of the prey seeking and catching that can resulting in high survival rates and growth rates in the juvenile stage of marine fish larvae (Kanazawa, 1997). Moreover, dietary DHA can be reflected in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) level. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were a group of phospholipid groups which normally found in the largest phospholipid fractions in fish tissue (Koven et al., 1993). Decreasing in PC and PE ratio suggested that the ability to regulate membrane fluidity are declining and may cause the fatty liver disease (Li et al., 2006). ARA is also considered as an important fatty acid. It can be involved in hormone stimulation as shown in the study in the *Sparus aurata* larvae (Bessonart et al., 1999), *Scophthalmus maximus* (Castell et al., 1994). Moreover, ARA could affect the production of eicosanoid which played the role in signal transduction in CNS, hormone secretion, immune system and biological activity as summarised in Wolfe (1982), Wolfe and Coceani (1979), White and Hagen (1982), Needleman et al. (1986), Samuelsson et al. (1987), Giles and Leff (1988) and Kitamura et al. (1989).

The fatty acid contents such as palmitic acid (16:0), oleic acid (18:1n-9) and linoleic acid (18:2n-6) particularly observed as high contents in the zooplankton such as in wild krill (Chang, 2000), white shrimp (Coutteau et al., 1996), copepods (Nanton and Castell, 1998) brine shrimp and rotifers (Otero-Ferrer et al., 2010). As well as the present study, 16:0, 18:1n-9 and 18:2n-6 were observed as high content in all feeding group. Palmitic acid are particular abundant in fish oil. The higher palmitic acid in diet associated with the higher palmitic acid contents in *Hippocampus* sp. This parallel increasing of palmitic contents was similar to the previous study in *Clupea harengus*

and *Salmo gairdnerii* (Henderson and Almatar, 1989; Henderson and Sargent, 1984). However, the influences of this fatty acid on fish growth and development were unclear. However, the roles of palmitic acid have been studied in mammal species. According to the report by El-Assaad et al. (2003) and (Jungheim et al., 2011) suggested that PA could have a negative impact on mammals in terms of insulin resistance, mitochondrial dysfunction and decreased in offspring growth rate. In the case of oleic acid (18:1n-9) was observed as high content in all feeding group. The study in Atlantic salmon showed that 18:1n-9 in diet could affected the secretion of triacylglyceral which was formed as energy stored in adipose tissue (Vegusdal et al., 2005). In the case of linoleic acid (18:2n-6), it could be transformed into arachidonic acid (ARA, 20:4n-6). ARA was the precursor of thromboxane could affected the platelets stimulation that was study in human (Kinsella et al., 1990).

In the case of other diet composition, the protein contents in the diets in the present study showed the similar content in the *Mysis* spp., the shrimp larvae and the enriched-copepod of 5.15 g 5.32 g and 3.89 g/100g ww, respectively. Protein contents are necessary for protein synthesis in fish tissues and also relating to the immune system by enhancing the phagocytes functions (Sitjà-Bobadilla et al., 2005). However, there were a limited study in terms of the total protein on fish growth and development. On the others hand, protein fraction referred to amino acids on growth, which was occurred in several study. Alanine and aspartate play some important roles as glucogenic precursor and provided the energy substrate for fish (Mommensen et al., 1980). Within the arginine, mostly found in protein which plays the important roles as endocrine regulators and also function in reproductive function (Jobgen et al., 2006; Yao et al., 2008).

Feeding Treatments on Gonadal Development of Hippocampus sp.

The reproduction in fish is affected by several environmental factors and diet nutritions. These have been studied in some adult fish e.g. *Oncorhynchus mykiss* (Fernández-Palacios et al., 1995), *Pagrus major* (Watanabe et al., 1991), *Sparus aurata* (Robaina et al., 1995). Nevertheless, there were a few studies in terms of gonadal development in the juvenile fish. However, the present study in juvenile fish could

primarily demonstrate the gonadal development which related to the diet composition in different preys.

Under the study in tissue level, *Hippocampus* sp. ovary was observed in paired that lied posteriorly in the coelomic cavity between kidney and posterior intestine. The ovary of *Hippocampus* sp. composed of several developing oocytes, which this feature was considered to be asynchronous developmental oocyte, that is similar to the reported in other seahorse species (Novelli et al., 2015; Poortenaar et al., 2004; Selman et al., 1991) and some fish in Syngnathids (Anderson, 1968; Begovac and Wallace, 1987). In this study, the developing oocytes in each stage also exhibited the differences and variant in sizes that similar to the reported by Poortenaar et al. (2004) and Selman et al. (1991).

In the case of gonadal differentiation and oogenesis, the ovary of juvenile *Hippocampus* sp. fed with *Mysis* spp. and shrimp larvae were observed first observed 11 DAB while the enriched copepod-fed group was observed in 17 DAB. The *Hippocampus* sp. fed with *Mysis* spp. exhibited the short duration of oocyte differentiation which was founded SGs in 31 DAB. Within the *Hippocampus* sp. fed with the shrimp larvae was founded the oocyte in oil droplets and cortical alveoli stage while this stage was not appeared in the *Hippocampus* sp. fed with enriched copepod. The study in this case could imply that the differences in HUFA may affect the duration of oocyte differentiation. There were no previous study reported the effect of diet on oocyte differentiation. However, the previous reported of Novelli et al. (2015) in *Hippocampus reidi* fed with the *Artemia nuplii* revealed the oocyte differentiation in the oil droplets and cortical alveoli stage at 31 DAB. The other dietary effect on gonadal development were usually revealed in the case of the egg quality and the egg numbers. (Bruce et al., 1999; Izquierdo et al., 2001; Watanabe et al., 1984; Woods and Valentino, 2003).

In terms of the dietary essential fatty acid which influenced the reproductive endocrine system. The short duration of oocyte differentiation in this study probably related to the endocrine regulation. The *Hippocampus* sp. obtained the high HUFA contents in *Mysis* spp. and shrimp larvae probably affected the shorter duration of

oocyte differentiation. Recent study by Tran (2015) strongly demonstrated that HUFA especially DHA and palmitic acid could induce an increasing in mRNA expression of Gonadotropin-releasing hormone (GnRH) that result in higher GnRH production. GnRH is produced from hypothalamus and could potentially result in an increasing in estrogen and vitellogenin production via Hypothalamus-pituitary-gland axis. Estrogen and vitellogenin are hormones that can regulate reproductive system. Estrogen (E2) is produced during gonadal differentiation which play an important role in the ovarian differentiation (Nakamura et al., 2003). High E2 also associates with vitellogenin levels (Mañanós et al., 1997). Thus, decreasing in E2 in plasma also resulting in the slow development of oocyte (Cerdá et al., 1994). Vitellogenin is also associated with the oocyte growth which was suggested by (Wallace, 1985), thus the deficient HUFA could affect lowering in vitellogenin content in serum (Fremont et al., 1984). They were less data in the case of effects of 16:0. However, the reported by Marei et al. (2010) in the bovine revealed the effect of 18:2n-6 increase the follicular size and it may play the role of oocyte maturation (Marei et al., 2010).

Referring to the previous reported in Syngathid, e.g. seahorse species and pipefish, showed the sex ratio in adult, which is 1:1 in *Hippocampus kuda* (Job et al., 2002) and pipefish with 1 male : 2 female ratio (Vincent et al., 1995). However, sex differentiation in Syngnatids has no previously reported. Nevertheless, the ovaries were observed as the only gonad in this study, which is similar to the report in *Hippocampus reidi* Novelli et al. (2015). The reproductive strategies, in terms of sex differentiation from this study could not confirmed because they probably performed under undifferentiated gonochroristic gonad (ovary firstly observed then differentiated into ovary and testis) or protogenic hermaphroditism (ovary firstly observed and then differentiated into testis).

Surprisingly, only ovary presented, not testes, in all samples by the histological observation. This probably may be resulted from several possible and unexpected factors. The first reason probably may be the temperature sex determination in the sea horse. The present experiment occurred during the rainy season which has the lower temperature (27.5 °C, personal measurement) less than the others season (29.5 °C, www.seatemperature.org/asia/thailand). Thus, the effect of different temperature is

likely related to sex-determination mechanism of the seahorse during the sexual differentiation. The disruption of the normal sex differentiation caused by variation in temperature probably affected the population sex ratios which reported in the study of *Menidia menidia* (Conover, 1984) and *Oreochromis aureus* (Mair et al., 1997). The second reason may be resulted from lack of genetic variation due to inbreeding of the single parent in the experiment. This may lead to the deviation of sex ratios in the offspring. The similar results in *Oreochromis niloticus* were also reported by Abucay et al. (1999). Moreover, the offspring sex ratios may be affected by the parental condition such as stresses. Thus they could lead to the biased sex ratio as reported in *Gallus gallus* (Parker, 2002) The third reason is like to be from the pollutants in the water under the captive condition. This could lead to decreasing in reproductive functions, reproductive fecundity and also exhibited the homologue reproductive organ in the population (Mensink et al., 1996). Furthermore, the pollutants may be an endocrine disruption (Taylor and Harrison, 1999) and can inhibit on reproductive biology (Kime, 1995). One of the pollutants is dioxin (2, 3, 7, 8-tetrachlorodibenzo-p-dioxin, TCDD). TCDD is one of the major pollutants from industries and incinerations, which are accumulated in the water and dispersed in the air. TCDD can cause reproductive and developmental malfunctions. These results have been reported, for example, lowering in male to female sex ratio in human offspring (Mocarelli et al., 1996). Moreover, the study in mice that obtained dioxin showed a decreasing in male to female ratio (Ishihara et al., 2007). Therefore, TCDD probably cause declining in gonadal development and sex ratio of *Hippocampus* sp.. This could be supported from the data by the Ecological Alert and Recovery (Thailand) (<http://earththailand.org>). The data indicate that river and off-shore water adjacent to the Phuket incinerator contained TCDD and other heavy metals that may be released from the industries and incinerations nearby (Campaign for Alternative Industry Network (CAIN), March 2006). The PMBC, which place that *Hippocampus* sp. nursery, is far from the incinerator only 6 kilometer as shown in Fig 5-1. Therefore, the *Hippocampus* sp. in the captive condition sex bias may be resulted from the pollutants in the water current.

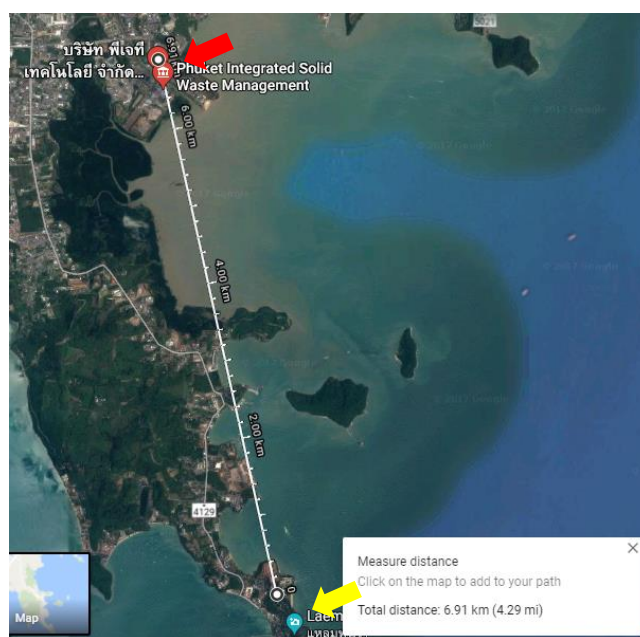


Figure 5-1 The location of the PMBC (*yellow arrow*) and the phuket incinerator (*red arrow*) which is around 7 kilometer distance.

Since it remains hard to predict the bioaccumulation in fish. Further investigations on tissue analysis, molecular studies, physiology of endocrine biomarker, hematological measurements and environmental pollutant measurement are still needed to improve *Hippocampus* sp. health in the future.

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APPENDIX



จุฬาลงกรณ์มหาวิทยาลัย
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VITA

Mister Thatpon Kamnurdnin was born on September 3rd, 1991 in Phuket, Thailand, which is my hometown. He finished his school from Phuket wittayalai in 2009. He received the Bachelor's Degree of Biology Science from Faculty of Science, Srinakarinwirot University in 2013. At present, he is a graduate candidate in Master's Degree in Marine Science, Faculty of Science, Chulalongkorn University.





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