นิเวศวิทยาการกินอาหารของปลาบู่ใส *Neostethus lankesteri* Regan, 1916 ในศูนย์ศึกษาเรียนรู้ระบบนิเวศป่าชายเลนสิรินาถราชินีและแม่น้ำปราณบุรี จังหวัดประจวบคีรีขันธ์

นางสาวอัมภรณ์พรรณ พลาศัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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FEEDING ECOLOGY OF PRIAPIUMFISH *Neostethus lankesteri* Regan, 1916 IN SIRINART RAJINI MANGROVE ECOSYSTEM LEARNING CENTER AND PRANBURI RIVER, PRACHUAP KHIRI KHAN PROVINCE

Miss Amphornphan Palasai

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อัมภรณ์พรรณ พลาศัย : นิเวศวิทยาการกินอาหารของปลาบู่ใส *Neostethus lankesteri* Regan, 1916 ในศูนย์ศึกษาเรียนรู้ระบบนิเวศป่าชายเลนสิรินาถราชินีและแม่น้ำปราณบุรี จังหวัดประจวบคีรีขันธ์ (FEEDING ECOLOGY OF PRIAPIUMFISH *Neostethus lankesteri* Regan, 1916 IN SIRINART RAJINI MANGROVE ECOSYSTEM LEARNING CENTER AND PRANBURI RIVER, PRACHUAP KHIRI KHAN PROVINCE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: เจษฎ์ เกษตระทัต, 70 หน้า.

ปลาบู่ใสชนิด *Neostethus lankesteri* เป็นปลากลุ่มเด่น และถูกตั้งสมมุติฐานว่าเป็นตัวเชื่องโยงระหว่างผู้ผลิตลำดับแรกกับผู้บริโภคที่สูงกว่าในระบบนิเวศปากแม่น้ำปราณบุรี จังหวัดประจวบคีรีขันธ์ อย่างไรก็ตามบทบาทหน้าที่และการใช้ประโยชน์จากพื้นที่ของปลาบู่ใสชนิดนี้ไม่เคยมีการรายงาน ในการศึกษานี้ใช้วิธีศึกษาองค์ประกอบอาหารในกระเพาะ สัณฐานวิทยาและมิญชวิทยา และการเกิดเซลล์สืบพันธุ์ (gametogenesis) เพื่อตรวจสอบการใช้ประโยชน์จากพื้นที่ของปลาบู่ใสชนิดนี้ ตัวอย่างปลาบู่ใส 30 ตัวถูกเก็บโดยอวนจับลูกปลาทุกเดือนจากสองพื้นคือ ปากแม่น้ำปราณบุรี และศูนย์ศึกษาเรียนรู้ระบบนิเวศป่าชายเลนสิรินาถราชินี (SRMELC) ระหว่างฤดูมรสุมตะวันออกเฉียงเหนือ และฤดูมรสุมตะวันตกเฉียงใต้ ผลการศึกษาองค์ประกอบอาหารในกระเพาะพบอาหาร 4 กลุ่มคือ ไดอะตอม ไดโนแฟลเจลเลต สาหร่าย และแพลงก์ตอนสัตว์ ดัชนีความสำคัญของอาหาร (IRI) แสดงให้เห็นว่า ไดอะตอมเป็นองค์ประกอบหลักในกระเพาะ รองลงมาคือแพลงก์ตอนสัตว์ในพื้นที่ปากแม่น้ำปราณบุรี ในทางตรงกันข้ามแพลงก์ตอนสัตว์เป็นองค์ประกอบหลักในกระเพาะรองลงมาคือไดอะตอมในพื้นที่ศูนย์ศึกษาเรียนรู้ระบบนิเวศป่าชายเลนสิรินาถราชินีของทั้งสองฤดู นอกจากนี้ปลาบู่ใสชนิดนี้ตำแหน่งปากอยู่ด้านบน (superior mouth) ประกอบด้วยฟันเขี้ยว และมีฟันรูปร่างแบบเขี้ยวบริเวณคอหอยจำนวนมาก สัดส่วนความยาวลำไส้ต่อลำตัวเท่ากับ 0.55 ผลการศึกษามิญชวิทยาของทางเดินอาหารในปลาบู่ใสชนิดนี้แสดงให้เห็นว่าปลาชนิดนี้ไม่พบโครงสร้างของกระเพาะอาหาร โดยมีลำไส้แบ่งออกเป็นสามส่วนคือ ลำไส้ส่วนต้น ลำไส้ส่วนกลาง และลำไส้ส่วนท้าย พบเซลล์กอบเบลท (Goblet cell) แทรกอยู่ในเยื่อบุผิวจำนวนมากและทำปฏิกิริยากับสีย้อมเพอริโอดิกเอสิดชิฟฟ์ (Periodic Acid Schiff) การศึกษาการเกิดเซลล์สืบพันธุ์ในเพศเมียของปลาบู่ใสชนิดนี้พบว่ารังไข่ระยะโตเต็มวัยมีการพัฒนาแบบไม่พร้อมกัน (asynchronous development oocyte) ประกอบไปด้วยการพัฒนาสองระยะคือ ระยะเริ่มแรก (primary growth phase) และระยะที่สอง (secondary growth phase) ทั้งยังพบระยะหลังปล่อยไข่ (post-ovulatory phase) ด้วยเช่นกัน การศึกษาการเกิดเซลล์สืบพันธุ์ในเพศผู้พบว่าเป็นแบบ restricted spermatogonial type การพัฒนาของสเปิร์มแบ่งออกเป็นสามระยะคือ ระยะ spermatogonial ระยะ spermatocyte และระยะ spermiogenetic โดยภาพรวมของการศึกษาองค์ประกอบอาหารในกระเพาะ สัณฐานวิทยาและมิญชวิทยา และการเกิดเซลล์สืบพันธุ์ การศึกษานี้เสนอว่าปลาบู่ใสชนิด *N. lankesteri* เป็นปลาที่มีการกินอาหารแบบเฉพาะเจาะจงต่อแพลงตอนสัตว์ และเข้ามาใช้ประโยชน์จากระบบนิเวศปากแม่น้ำปราณบุรีเพื่อเป็นแหล่งหาอาหารและแหล่งสืบพันธุ์วางไข่

ภาควิชา วิทยาศาสตร์ทางทะเล ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาศาสตร์ทางทะเล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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AMPHORNPHAN PALASAI: FEEDING ECOLOGY OF PRIAPIUMFISH *Neostethus lankesteri* Regan, 1916 IN SIRINART RAJINI MANGROVE ECOSYSTEM LEARNING CENTER AND PRANBURI RIVER, PRACHUAP KHIRI KHAN PROVINCE. ADVISOR: JES KETTRATAD, Ph.D., 70 pp.

The priapiumfish, *Neostethus lankesteri* is a dominant species of the Pranburi River ecosystem. It was hypothesized as a link between primary producer and higher consumer of the Pranburi River Estuary ecosystem, Prachuap Khiri Khan Province. However, the roles and utilization of *N. lankesteri* in the ecosystem have not been reported. In this study, I used gut content analysis and morpho-histology of the digestive tract and the gametogenesis to examine the habitat utilization of this fish. Thirty individuals of *N. lankesteri* were collected monthly by larval trawl from two sites: Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center (SRMELC), during Northeast Monsoon and Southwest Monsoon season. Based on the gut content of *N. lankesteri*, food items were categorized into four groups: diatoms, algae, dinoflagellates, and zooplankton. The index of relative importance, IRI showed that diatoms were a major component followed by zooplankton at Pranburi River estuary, whereas zooplankton was a major component followed by the diatoms at SRMELC during the two seasons. Moreover, *Neostethus lankesteri* has a superior mouth with canine teeth. The pharyngeal toothplates were numerous with canine-shaped. The intestine coefficient of *N. lankerteri* was 0.55. The results of the histology of the digestive tract in *N. lankesteri* showed that they were stomachless, which the intestine consisted of three regions: anterior intestine, middle intestine, and posterior intestine. Goblet cells were also observed among epitheliums and positively reacted to Periodic Acid Schiff staining method. The study of the gametogenesis of female *N. lankesteri* revealed that the mature ovary in these fish was an asynchronous development oocyte type which consisting of two phases: primary growth phase and secondary growth phase. Post-ovulatory phase was observed in the sampling periods as well. In male *N. lankesteri*, the mature testicular parenchyma was observed. The spermatogenetic stage was divided into three phases including spermatogonial, spermatocyte and spermiogenetic phases. Overall, based on gut content, morpho-histology of the digestive tract, and gametogenesis, I suggest that *N. lankesteri* is zooplankton feeder specialist (*sensu lato*) and utilized the Pranburi River estuary ecosystem for both feeding ground and spawning ground.

Department:	Marine Science	Student's Signature	.....
Field of Study:	Marine Science	Advisor's Signature	.....
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CHAPTER 1

INTRODUCTION

The priapiumfish, *Neostethus lankesteri* Regan, 1916 belongs to the Family Phallostethidae. In male, the pelvic fin of this species is modified into a reproductive organ called a "priapium". This species has a wide-range distribution from Indonesia, Malaysia, Singapore to Thailand (Myers, 1928; Parenti, 1984). In Thailand, the priapiumfish was found along the rivers and estuaries, such as Pranburi River estuary (Paphavasit *et al.*, 2014). The previous study also reported that the priapiumfish is a dominant species in fish communities within Pranburi River estuary. However, the information concerning the habitat utilization is still lacking. In order to understand the habitat utilization of this species, the fundamental knowledge; e.g. feeding ecology and reproductive biology, is required.

Feeding ecology of priapiumfish is important to be explored since it offers a better understanding of their functional role within the ecosystems. Furthermore, this knowledge provides better understanding not only in their trophic interactions in aquatic food webs (Garvey, James E *et al.*, 1998; Zanden *et al.*, 2000) but also in their resources partitioning in the ecosystem (Guedes & Araújo, 2008; Ross, 1986). The study of fish feeding habits and the analysis of gut content has become a standard component for the trophic analysis in the ecosystem (Hyslop, 1980). The feeding and histology aspects of priapiumfish are both essential; the characteristic of histology regarding the digestive tract could describe the differences of its feeding habits (Nazlić *et al.*, 2014; Raskovic *et al.*, 2011; Silva *et al.*, 2012). This information eventually leads to better comprehension, and also demonstrates how the priapiumfish utilizes the estuary. Moreover, the reproductive biology will provide an important piece of information such as the spawning season, which could be used for developing the conservation strategies and the management plans. The aspect of gonadal histology, in order to describe the development of the gonad, and to provide more clarification on how this species utilizes the estuary as its reproductive area.

The goal of this research is to determine how this fish utilizes the Pranburi River estuary by using the gut content analysis, morpho-histology of the digestive tract, and gonadal histology.

Objectives

1. To study the gut contents of *Neostethus lankesteri* Regan, 1916
2. To study the morpho-histology of digestive tract of *Neostethus lankesteri* Regan, 1916
3. To study the gonadal histology of *Neostethus lankesteri* Regan, 1916

CHAPTER 2

LITERATURE REVIEW

The priapiumfish *Neostethus lankesteri* Ragan, 1916

Taxonomy and Morphology

The priapiumfishes belong to the Order Atheriniformes and the Family Phallostethidae (Parenti & Louie, 1998). Male phallostethids have a unique intromittent organ called "the priapium", thus the fishes of this family are commonly called "priapiumfish" (TeWinkel, 1939). The family Phallostethidae are classified into four genera; *Gulaphallus*, *Neostethus*, *Phallostethus* and *Phenacostethus* (Hureau & Monod, 1973; Nelson & Platnick, 1981; Parenti, 1989; Parenti, 1996; Parenti & Louie, 1998).

The priapiumfish, *Neostethus lankesteri* Regan, 1916 belong to genus *Neostethus*. This fish is small (can reach to 3.7 cm standard length), laterally compressed, eyes large, superior mouth, moderate gape, slightly and nearly transparent. They have cycloid scale, small to moderate, and scattered minute dark brown to black melanophores on head and body (Parenti, 1989; Roberts, 1971; Shibukawa *et al.*, 2012). *Neostethus lankesteri* have small teeth on distal portion of premaxilla, falcate pectoral fins, emarginated caudal fin, anterior anus and urogenital openings and covered by papilla in females. In addition, they have one spine on first dorsal fin; second dorsal fin with 5 to 7 soft rays anal fin with 16 to 17 soft rays; pectoral fins with 9 to 13 soft rays and vertebrate 34-35 (Parenti, 1996).

Reproductive biology

In males, the pelvic fin is modified to be an intromittent organ for holding or clasping onto females and fertilizing their eggs internally (Parenti, 1989). It is called a "priapium" (Figure 2.1). The priapium is ventral bony projection on elongate first ctenactium (Breder & Rosen, 1966; Parenti, 1989). On the other hand, in female's reproductive biology, they have a large hook-liked, posteriorly projecting, urogenital

papilla (Parenti, 1989; TeWinkel, 1939). In addition, females do not give birth to live young, but instead lay fertilized eggs (Parenti, 1989).

Figure 2.1 Sexual dimorphism of *Neostethus lankesteri*: **(A)** male *N. lankesteri* showing a priapium organ (arrowhead), **(B)** female *N. lankesteri.*

Distribution

The phallostethids are found in both fresh and brackish water (TeWinkel, 1939). The habitat of this fish was commonly in the intertidal areas of mangrove forests and brackish-water. The priapium fish genus *Neostethus* distributed in Southeast Asia only (Myers, 1928; Parenti, 1984). First record of *Neostethus* in Thailand was reported by Myers (1937) under the name *N. siamensis* from the estuary of Chantaburi River, Southeast Basin, Thailand. Currently, in Thailand, the genus *Neostethus* have been reported in form the brackish water in Phetburi River (Kunlapapuk *et al.*, 2012a) and Pranburi River (Paphavasit *et al.*, 2014).

The priapiumfish relate to Pranburi River Estuary

Mangrove forest provides diversity and abundance of food sources. Mangrove forests act as the barrier reducing erosion from natural disaster such as currents waves, and tides. The complexities of root system of the mangroves and the turbidity in the estuary provide attractive habitats for several fishes and other organisms utilizing food, nursery, and shelter from predators (Laegdsgaard & Johnson, 2001).

Pranburi River estuary and mangrove forest confronted with problems with forest deforestation, intensive shrimp farming, and urban intrusion to forest area in the past 20 years. In the year of 2002, Sirinaj Rajini Mangrove Ecosystem Learning Center (SRMELC) was established in order to retain and recover the mangrove forest (Paphavasit *et al.*, 2014). The assessment of biodiversity has begun at that time. Many researchers were assigned to assess and monitor the forest diversity. Paphavasit *et al.* (2014) evaluated the diversity and productivity of Pranburi River estuary. The study revealed the progresses in the recovery of the mangrove productivity when compared to the year prior to the reforestation. The salinity in the SRMELC was higher than the Pranburi River estuary year round. The authors suggested that it was because of low water circulation and low exchange of water masses in the river. The Pranburi estuary was brackish throughout year as a consequence of low freshwater input. The freshwater flow was controlled by the Pranburi Dam. The salinity regime in the river ranged 19-32 psu throughout the years. The deposition of the liters within the mangrove forest and estuary creates a detrital based food web, often considered to explain why many species

of organism utilize the mangrove ecosystem (Chaves & Bouchereau, 2000; Wongchinawit, 2007). Paphavasit *et al.* (2014) found several fishes utilized mangrove for different purposes e.g. feeding ground, nursery area and shelter area. Fishes in estuary ecosystem can be categorized into four major groups according to life history: estuarine species, marine migrants, anadromous species and freshwater migrants (Blaber, 2000).

1. Estuarine species can complete their life in the estuary. Fishes in this category can tolerate a wide range of salinities (euryhaline) and turbidities (Whitfield, 1980). Estuarine species in Thai mangrove forests consist of fishes in the families Engraulidae, Clupeidae, Leiognathidae, Gerreidae, Eleotridae and Gobiidae (Satapoomin & Poovachiranon, 1997; Somkleeb *et al.*, 2001; Tongnunui, 1997; Vitheesawat & 1999).

2. Marine migrants are marine species that spend time in both sea and estuaries. They utilize mangrove when they are adults and they use the mangrove mainly searching for food. The example of marine migrants are *Dasyatis sephen* (Shokita, 2000), *Chirocentrus dorab* (Blaber *et al.*, 1995), and *Liza grandisquamis* (King, 1984). Post larvae and juveniles of several species of Engraulidae and Clupeidae moved into the estuaries and shallow water in order to exploit their foods (Chaves & Bouchereau, 2000; Robertson *et al.*, 1988; Satapoomin & Poovachiranon, 1997).

3. Anadromous species are the species that ascend the rivers to spawn in freshwater where the juveniles disperse to the estuarine and marine habitat. The example of fishes in this category are fishes in the families Salmonidae (Bruno *et al.*, 1986) and Ophichthidae (Riede, 2004).

4. Freshwater migrants are freshwater fishes that move varying distance down the estuaries, sometimes to spawn, but usually return to freshwater to spawn. Freshwater migrants regularly found in moderate numbers in estuaries and their distribution can extend beyond the oligohaline sections of estuaries system. Small larvae or early juveniles often grow rapidly and leave the system before maturing in the adjacent estuary such as fish in the family Toxotidae (Blaber, 2000).

According to Paphavasit *et al.* (2014) priapiumfish, *Neostethus lankesteri*, was found dominantly along to Pranburi River estuary and in SRMELC. *Neostethus lankesteri* was hypothesized as the link between primary producer and higher consumer which feed on zooplankton e.g. copepods and mollusk larvae (Mok & Munro, 1991).

Neostethus lankesteri was believed to feed according to Optimal Foraging Theory (Wootton, 2012) assume that food is chosen to maximize the profitability income. Moreover, the presence of a large number of *N. lankesteri* in the area is an indicator of the importance of this fish in the food webs. Unfortunately, although this fish is usually found dominantly in the ecosystem, the understanding of its roles and habitat utilization is little known. Therefore, the feeding ecology of this priapiumfish in relation to feeding structure morpho-histology as well as reproductive biology is needed to understand.

Feeding ecology of fish

Feeding ecology is important for understanding the functional role of the fish within the ecosystems. Not only does this knowledge helps understanding trophic interactions in aquatic food webs (Garvey, James E *et al.*, 1998; Zanden *et al.*, 2000) but it also helps in understanding resources partitioning, trophic ecology, ultimately ecosystem dynamic (Guedes & Araújo, 2008; Ross, 1986). The Optimum Foraging Theory (OFT), was needed to determine feeding behavior of fishes. It is correlate to forage most benefits rate of energy income minus the energy costs of forage their food sources (Gerking, 2014). The terms generalist, specialist, and opportunist are proposed to describe food habits by Gerking (2014). Generalists eat a broad spectrum of foods in terms of prey species or microhabitats in which the prey live. Specialist implies a diet restricted to relatively small number of species. Specialists are selected the prey items accordingly to the optimal foraging theory with emphasis on nutrition and energy. Opportunists are, obviously, one who takes advantage of opportunities which can switch from one food source to another.

The study on the feeding ecology of fishes usually used stomach content as a method to determine feeding habits of fishes. The descriptive of feeding information have been done by numerous methods to evaluate the feeding ecology. For examples, gut content analysis (Cortés, 1997; Hynes, 1950; Hyslop, 1980), stable isotope analysis (Fanelli *et al.*, 2009; Graham *et al.*, 2007) and fatty acid analysis (Young *et al.*, 2010). Normally, the gut content analysis was the primary used to study the stomach content in teleost. Due to its simple procedure and it provides good results. Many studies were used the gut content to determine several fishes such as *Mugil cephalus*, *Selene*

peruviana and *Eucinostomus currani* (Ramírez-Luna *et al.*, 2008) and *Rastrelliger kanagurta* (Sivadas & Bhaskaran, 2009). The stomach/gut content analysis was co-evaluated by calculating the index of relative importance, IRI to accurately interpret the result from the stomach/gut content. The index of relative important (IRI) was calculated from three aspects of prey item that fish fed: number, volume, and frequency of prey item (Hyslop, 1980; Pinkas *et al.*, 1971). The IRI has the formula as follows: IRI = %O × (%N + %V), where percentage by number (%N), percentage by volume (%V) and percentage by frequency of occurrence (%O).

Research hypothesis

Since the previous study stated that *N. lankesteri* fed on zooplankton mainly copepods (Mok & Munro, 1991). But the knowledge of feeding ecology and habitat utilization in Thailand, especially in the Pranburi River estuary where the *N. lankesteri* was found dominantly, are still not understood. While other genera within the family Phallostethidae are found in both freshwater and saltwater. Hence, two alternative life history (freshwater migrants or partial resident and true estuarine species) could be predicted form the distribution of the family. However, the genus *Neostethus* have been reported only form the brackish water. Hence, it is likely that they are true estuarine species. Therefore, I hypothesized that *N. lankesteri* utilized the Pranburi River estuary ecosystem and Sirinaj Rajini Mangrove Ecosystem Learning Center as a feeding ground and a spawning ground. Therefore, in order to understand the feeding ecology as well as the reproductive biology of this species. The study of feeding ecology of *N. lankesteri* and the reproductive biology in the aspect of gametogenesis was performed. The result of this study will provide in depth knowledge of habitat utilization of *N. lankesteri* in the ecosystem.

CHAPTER 3

GUT CONTENT AND HISTO-HISTOCHEMISTRY OF THE DIGESTIVE SYSTEM OF *Neostethus lankesteri* Regan, 1916 AND RELATIONSHIP WITH FEEDING TYPE

Introduction

Neostethus lankesteri belonging to Family Phallostethidae is commonly distributed in Southeast Asia particularly Thailand such as Chantaburi River (Myers, 1937), Phetchaburi River (Kunlapapuk *et al.*, 2012b) and Pranburi River (Paphavasit *et al.*, 2014). Based on ecological important feature, it is possible that this fish may play a key role in linking the primary producer and the secondary consumer. Most of previous literatures suggested that Phallostethid are carnivores, which feed mainly on insects (Munro & Mok, 1990; Villadolid & Manacop, 1934). Additionally, Mok and Munro (1991) reported copepods are the main food item of *Neostethus bicornis* and *N. lankesteri* found in Singapore. However, this study suffered from low sample size (n=17) and lacked of season variation consideration in their sampling regime. Furthermore, fishes within the same genus and within same species have shown to select different prey items in different habitats (Kaifu *et al.*, 2013). Therefore, fundamental knowledge on the characterization of feeding type of *N. lankesteri* in Thailand is required, which it has never been reported.

Feeding ecology is important for understanding the functional role of the fish within their ecosystems. This information will contribute to develop the management strategies of fish species (Melo Germano *et al.*, 2014; Rodrigues & Menin, 2005) because it also shows the relationship among trophic levels in food webs and food chain (Garvey, J. E. *et al.*, 1998; Vander Zanden *et al.*, 2000) as reported in a variety of fishes such as *Terapon jarbua* (Manoharan *et al.*, 2012) *Cyprinion mhalensis* (Ahmad *et al.*, 2013) and *Clarias gariepinus* . Several methods have been used to determine the feeding ecology of fish, but the gut content analysis is recommend and emphasized as the best important method (Hyslop, 1980). It is relatively simple procedure and provides good results (Ducommun *et al.*, 2010; Kaifu *et al.*, 2013; Wootton, 2012). A

previously described procedure of the gut content was used to determine in several fishes such as *Mugil cephalus*, *Selene peruviana* and *Eucinostomus currani* (Ramírez-Luna *et al.*, 2008) and *Rastrelliger kanagurta* (Sivadas & Bhaskaran, 2009). In fact, the characterization of the feeding aspect is studied to ensure in parallel to histological aspect. In this study, the gut content analysis and histological structure of the digestive system in *Neostethus lankesteri* Regan, 1916 from Thailand were investigated**.**

Materials and Methods

Fish collection and study area

Healthy adult *Neostethus lankesteri* with standard length (SL) of 2.53±0.31 (mean±SD) cm were collected by larval trawl from two sites; Pranburi River Estuary (ST1, 12°24'15.8" N, 099°58'25.6" E, ST2 12°24'21.6" N, 099°58'37.1" E, ST3 12°24'08.5" N, 099°59'00.2" E) and Sirinart Rajini Mangrove Ecosystem Learning Center, SRMELC (ST4, 12°23'43.52" N, 099°58'49.45" E, ST5, 12°23'53.77" N, 099°58'55.98" E, ST6, 12°23'53.52 "N, 099°58'53.0" E), Thailand from March 2015 to February 2016 which covered the two seasons (Northeast monsoon and Southwest monsoon season). Thirty individuals/ station/ month (Total random sampling of 2160 individuals) were collected from six localities (Figure 3.1). All fish were euthanized by a rapid cooling shock protocol (Wilson *et al.*, 2009). The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review No. 1523005).

Analysis of gut content

All specimens were fixed with Davidson's fixative (about 48 hrs) and then transferred to 70% ethanol to study the gut content. After dissecting, all samples of the digestive tract were examined and the intestine was measured to calculate the intestine coefficient (IC). The prey items were identified to detail according to the guidelines of Tomas (1997) and Casanova and Boltovskoy (1999) under stereomicroscopy (Olympus CX3 1 light microscope) and Dino Eye Piece AM-423C. The percentage by frequency of occurrence (%O), the percentage by number (%N) and the percentage by volume (%V), as described by Pinkas *et al.* (1971) and Hyslop (1980) were calculated. The index of relative importance (IRI) was used to describe the important of fish's diet and explain the feeding habit of fish (Cortés, 1997; Hyslop, 1980).

Gross anatomy and histological observation

Fixed 40 specimens *Neostethus lankesteri* with 2.44±0.33 SL cm (mean±SD) cm were dissected for both digestive tract (oral cavity to intestine) and accessory organs (liver and pancreas) under stereomicroscopy. All samples were investigated for their gross anatomy and subsequently subjected to standard histological techniques (Bancroft *et al.*, 2013; Presnell *et al.*, 1997). The paraffin blocks from specimens were sectioned at 6-7 μm thickness using the rotary microtome. Histological sections were routinely stained by Delafield's hematoxylin and eosin (H&E) and specially stained with Masson's trichrome stain for detection of fiber and connective tissue, periodic acid schiff (PAS) for the detection of glycoproteins and alcian blue pH 2.5 (AB) for detect of mucopolysaccharides (Bancroft *et al.*, 2013; Presnell *et al.*, 1997). The histological observation of the digestive tract and accessory organs of this species were assessed by using light microscope and photographed using Olympus CX31 light microscope mounted with a Canon EOS100d camera.

Data analysis

An analysis of variance (ANOVA) was used to examine the differences in preys composition and salinity in each habitat between Northeast Monsoon and Southwest Monsoon season, and used for examining the differences in the longitudinal folds length among different intestine regions. All of the statistic tests were done with program IBM SPSS statistics 22.

Results and Discussion

Analysis of gut content

In this study, the food items of the *Neostethus lankesteri* can be classified into four groups: diatoms (centric diatom and pennate diatom), algae, dinoflagellate (Noctiluca sp.) and zooplankton (e.g., copepods, cirripedia larvae and copepods nauplii) (Table 3.1). *Neostethus lankesteri* in this study showed variety of diets, the quantity of the prey items in the gut content from the Southwest monsoon were not significant differences from the prey items found in the Northeast monsoon. On the other hand, the preys items in gut content of this fish from Pranburi River estuary were significant different ($P<0.001$) from those that were from the Sirinart Rajini Mangrove Ecosystem Learning Center (SRMELC).

Table 3.1 Summary of prey items found in the gut contents of *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016.

	Northeast monsoon				Southwest monsoon			
	Pranburi River Estuary		SRMELC		Pranburi River Estuary		SRMELC	
	IRI	%IRI	IRI	%IRI	IRI	%IRI	IRI	%IRI
Diatoms	879071	89	60839	18	861981	92.1	112976	47
Algae	592	0.1	n/a	n/a	592	0.1	n/a	n/a
Dinoflegellate	926	0.1	n/a	n/a	135	0.0	29	<0.0
Zooplankton	101455	10	260840	81	73589	7.9	125597	52
Copepods	35921	3.7	199669	62	37777	4.0	90716	38
Mollusk larvae	58	<0.0	1031	0.3	1180	0.1	549	0.2
Cirripedia larvae	7960	0.8	8355	2.6	53	<0.0	2371	1.0
Nematode	9006	0.9	20084	6.2	9041	1.0	117	<0.0
Foraminifera	1.7	n/a	n/a	n/a	17	n/a	n/a	n/a
Copepods nauplii	15031	1.5	5214	1.6	7803	0.8	4040	1.7
Polycheate larvae	6028	0.6	n/a	n/a	4.6	n/a	n/a	n/a
Tintinids	2085	0.2	3429	1.1	16326	1.7	7493	3.1
Shrimp larvae	4493	0.5	765	0.2	4.4	n/a	328	0.1
Arrow worms	n/a	n/a	19658.9	6	690	0.1	n/a	n/a
Insects	4575	0.5	670	0.2	20	n/a	19026	8.0
Fish eggs	9734	1.0	278	0.1	611	0.1	873	0.4
Amphipod	6553	0.7	n/a	n/a	n/a	n/a	n/a	n/a
Ostracod	4.0	n/a	1155	0.4	n/a	n/a	6.3	<0.0
Crabs larvae	n/a	n/a	526	0.2	57	0.0	73.4	<0.0

Note: IRI = index of relative important, n/a = no data.

Mok and Munro (1991) reported that *N. lankesteri* fed mainly on zooplankton, especially copepods, in Sungei Buloh basin, Singapore. Phytoplankton was not found in their diets. They observed the gut content of 17 specimens of *N. lankesteri* and they found only zooplankton (copepods, mullusc larvae, cirripedia nauplii, foraminiferans, and decapod larvae) in the fish diet. They also did the study on *N. bicornis* (the closely related species of *N. lankesteri*). Based on 76 specimens, *N. bicornis* diet component contained only zooplankton as well. However, that diet composition from *N. lankesteri* in this study was different from Mok and Munro (1991). In this study the gut content of *N. lankesteri* from Pranburi River estuary from both seasons showed that the major component of the diet was diatoms (approximately 90%IRI) (Figure 3.1).

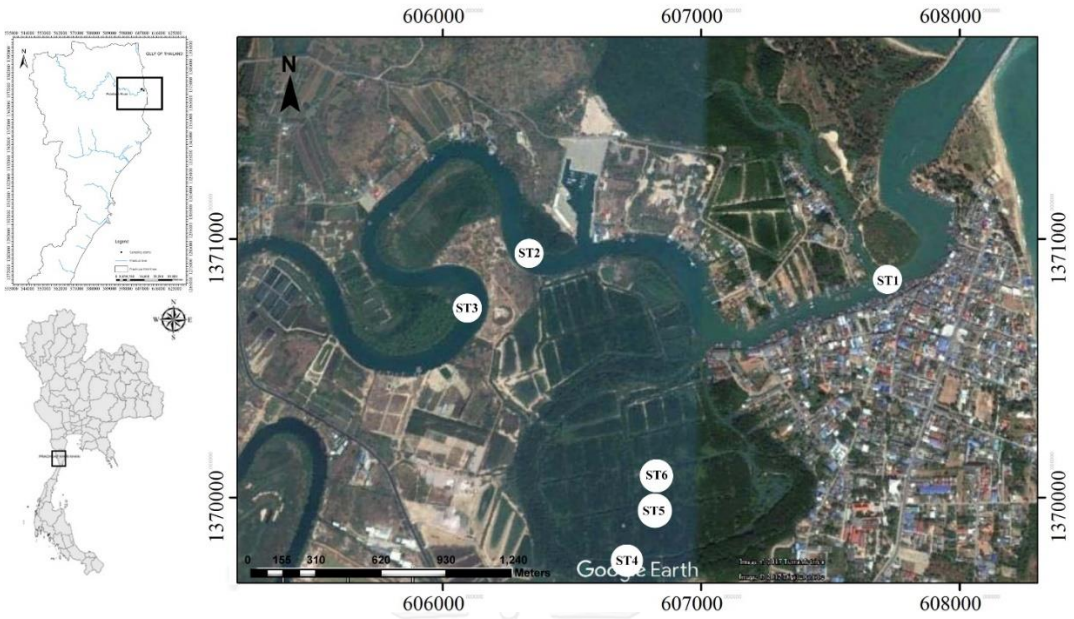

Figure 3.1 Sampling areas of *Neostethus lankesteri* in Pranburi River estuary (ST1-ST3) and Sirinaj Rajini Mangrove Ecosystem Learning Center (ST4-ST6), Pranchuap Khiri Khan Province.

Algae and dinoflagellate were also found in the guts content of *N. lankesteri* from this site. The Diatoms was found in high concentration in the Pranburi River estuary throughout the years. The portion of diatoms available in the water was approximately 10,000 cells per liter during both sampling seasons in this site (Figure 3.2). This is consistent with previous reported that the diatoms were the dominant phytoplankton in Pranburi River estuary in both seasons (Paphavasit *et al.*, 2014). In contrast, the diet component of *N. lankesteri* from SRMELC showed that the major component was zooplankton (81%IRI) which copepods contributed more than 62%IRI of total zooplankton component from the Northeast monsoon. During the southwest monsoon, zooplankton which copepods contributed approximately 38%IRI of total zooplankton component was the major component (52%IRI) (Table 3.1 and Figure 3.3).

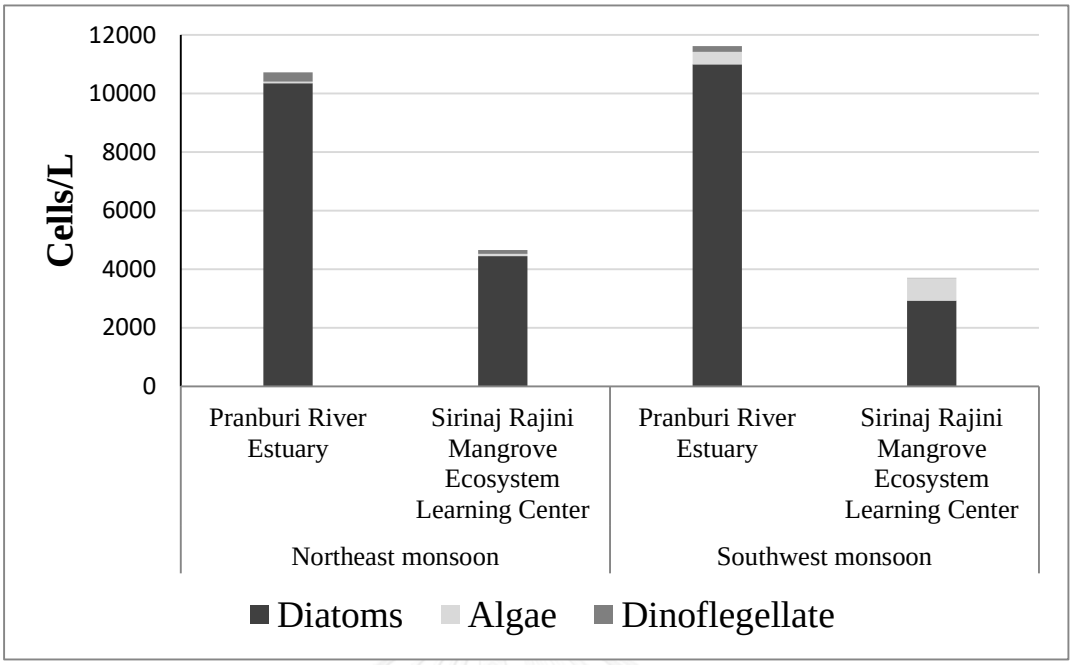

Figure 3.2 Availability of phytoplankton in water columns collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016.

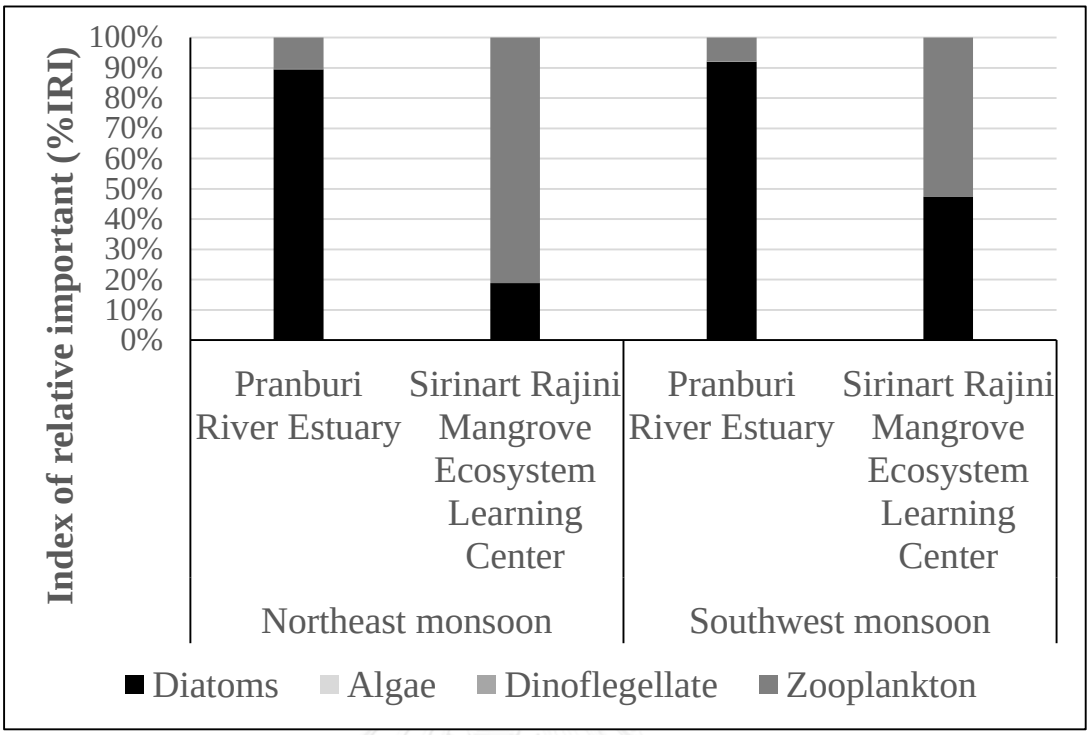

Figure 3.3 Food items found in the gut of *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during Northeast monsoon (2015-2016) and Southwest monsoon (2015).

When compared the diatom density between the Pranburi River estuary and SRMELC, the diatom density from SRMELC was significantly different (P=0.017) from the diatom concentration from Pranburi River estuary. The diatom density from SRMELC was less than the Pranburi River estuary (Figure 3.2). This is consistent with the study by Paphavasit *et al.* (2014). Since the diatoms were found in large amount in the water column of the two sites (Pranburi River and SRMELC). The dominant prey item in the gut content of *N. lankesteri* from Pranburi River estuary were the diatoms which is the same with the second highest abundance prey item in the gut content of *N. lankesteri* from the SRMELC (Figure 3.3). The occurrence of the diatom in the gut content was likely the contribution from incidental ingestion, which is the case that fish accidentally eat certain prey during the pursuit of target prey (O'brien, 1987). This scenario of incidental ingestion also occurred in Scorpaenid fish, *Centropogon australis* (Bell *et al.*, 1978). The authors stated that *C. australis* were not only found as macrophagic carnivore, but they also found seagrass and algae as minor food items in

the gut. The authors suggested that it was an accidentally consumed which seagrass and algae are attached to the prey during ingestion. In this study, the diatom in the gut content of *N. lankesteri* may show the same scenarios as previously reported. Therefore, I suggested that either the diatom in the gut content of *N. lankesteri* might be accidentally ingested or *N. lankesteri* might select directly on diatoms.

The differences of phytoplankton and zooplankton community between Pranburi River estuary and SRMELC in each seasons (Northeast monsoon and Southwest monsoon) may related to several factors e.g., nutrient and salinity. For example, the salinity of Pranburi River estuary and SRMELC was significant different (P=0.0001) (Figure 3.4). Salinity can affect plankton species assemblages (Schröder *et al.*, 2015). Qasim *et al.* (1972) also suggested that the relative abundance of phytoplankton in the estuary corresponded to the waters salinity changes. Water with low salinity supported a high abundance of phytoplankton. Many species of phytoplankton have their own optimal salinity range for cell division (Nagai & Imai, 1999). The diatom such as *Coscinodiscus* spp. has the optimal salinity for cell enlargement less than 30 psu (Nagai & Imai, 1999). The high abundance of the diatom from the Pranburi River estuary could be explained by the proper salinity condition (salinity ranged from 18-28.9 psu), while the SRMELC locality showed lower diatom concentration with salinity ranged from 28.1-32.1 psu which was not suitable for the diatom (Figure 3.4). Phytoplankton community has been reported to relate to local nutrient loads (Álvarez-Góngora & Herrera-Silveira, 2006; Thongdonphum *et al.*, 2014). The amount of nutrients discharged e.g. NH_4^+, $NO_2^- + NO_3^-$, and PO_4^{3-} into the water influenced the phytoplankton abundance. These nutrients were the crucial factors to phytoplankton growth and may led to phytoplankton bloom phenomena along the coastal zones (Thongdonphum *et al.*, 2013; Thongdonphum *et al.*, 2014).

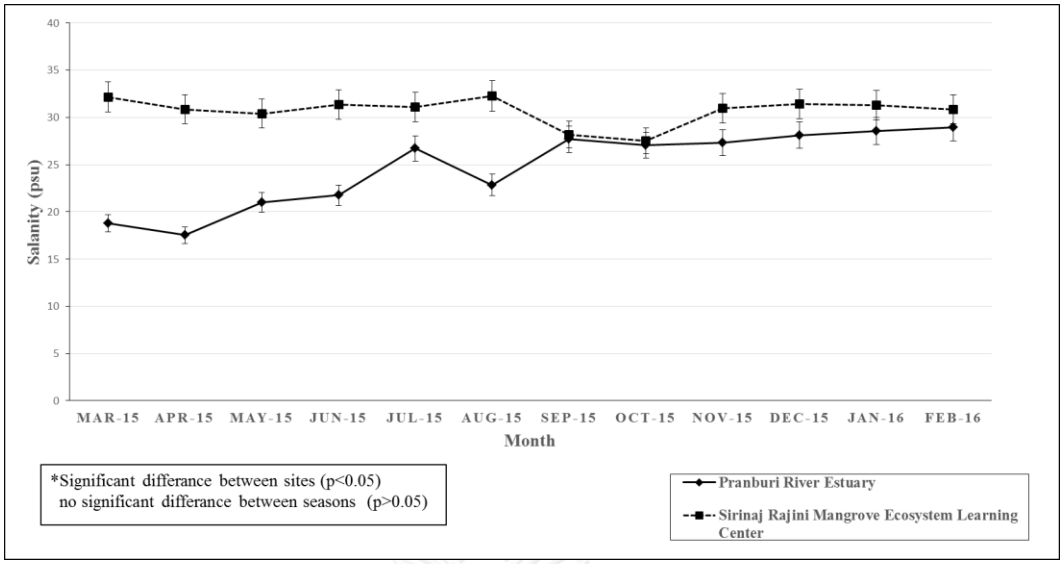

Figure 3.4 Salinity changes during sampling period (March 2015 to February 2016) from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center.

Thongdonphum and colleagues (2013) reported that the main discharged sources of nutrient loads of the Mae Klong River, Thailand was from sewage, domestic discharges, and agricultural and aquacultural utilization. This scenario is also similar to the situation in the presence study, the stations ST1, ST2 and ST3 which located at the Pranburi River estuary has high activities of discharged than the sites located in SRMELC. Even though, this study did not observed the parameter of any nutrients. However, the previous studied of Paphavasit *et al.* (2014) reported the range of the overall nutrients in Pranburi River estuary was slightly lower than that in SRMELC. The levels of $NO_2^- + NO_3^-$, $Si(OH)_4$, PO_4^{3-}, and NH_4^+ in Pranburi River estuary ranged from 0.04 to 15.51, 9.7 to 89.97, 0.38 to 3.46, and 2.38 to 13.28 μM, respectively. Whereas those nutrients in SRMELC ranged from 0.12 to 20, 5 to 105.47, 0.27 to 3.83, and 2.44 to 23.02 μM, respectively. However, they did not use statistical test to determine the differences. The case reported by Paphavasit *et al.* (2014) was the opposite scenario from the general idea that higher nutrients load will occur at Pranburi River estuary when compared to the mangrove forest (SRMELC). It was suggested that the low water exchange and slow circulation between mangrove forest and estuary was responsible for such pattern. They further explained that ammonia produced by defecation from organisms and the concentration of nitrite produced from bacterial

denitrification in low dissolved oxygen area created a nearly anoxic layers on the mangrove floors (Paphavasit *et al.*, 2014). Without water circulation, this process could produce high excess nutrient in the SRMELC. Water circulation dynamic in the estuary is important in transports nutrients between upper to lower estuary (coastal area), tidal water movements characterized by ebb and flood flows are act as a vehicle for the flux of inorganic nutrients between the different coastal ecosystems (Wolanski *et al.*, 1980). Coastal water circulation association with freshwater input affects the high nutrient distribution and related to phytoplankton productivity in coastal ecosystems (Kitheka *et al.*, 1996). Generally, in the area of well-circulation which helps the incoming flux of the high nutrients to the area which normally result in the increase in the concentrations of phytoplankton and zooplankton.

The opposite scenario is also tended to be true in poor-possessed water circulation area (Kitheka *et al.*, 1996; Skarðhamar *et al.*, 2007). The general scheme of the relationship between phytoplankton concentration and the rate of water circulation is similar to what was found in Paphavasit *et al.* (2014). Although they did not address this relationship, they found that the Pranburi River estuary which had high phytoplankton concentration has relatively better water circulation than the SRMELC which had relatively low plankton concentration. This is supported by previous studies, the diatoms was the dominant phytoplankton in the river mouth, which could be explained by the nutrients input by river discharges, sewage, and water circulation process (Kitheka *et al.*, 1996; Skarðhamar *et al.*, 2007; Thongdonphum *et al.*, 2014; Trobajo & Sullivan, 2010). Hence, the high abundant of the diatoms in the Pranburi River estuary could be explained by high nutrient loads induced by anthropogenic activities from both seasons and well-possessed water circulation. On the other hand, stations in SRMELC located neared by Pranburi River estuary had lower diatom concentration was probably due to lower nutrient load which was probably caused by the low water exchange rate between Pranburi river estuary and SRMELC (Paphavasit *et al.*, 2014). Therefore, the nature of the spatial distribution of the food sources which is normally not uniformly distributed may influent the difference of the composition of food in gut content (Bootsma *et al.*, 1996; Kruuk & Moorhouse, 1990). Based on the gut content of *N. lankesteri* and the amount of zooplankton and phytoplankton found in

its habitat, this study suggested that the feeding behavior of *N. lankesteri,* it could be a generalist feeder guild.

Digestive tract morphology

Neostethus lankesteri has superior mouth and highly protrusible. It is similar to what was found in *N. bicornis* (Mok & Munro, 1991). The average width and height of mouth gape was 1.25±0.02SD and 1.55±0.11SD mm, respectively. When compared to their dominant food items in the gut, the dimension of zooplankton and phytoplankton (copepods with of 0.18±0.08SD mm width, 0.58±0.32SD mm length and phytoplankton, *Coscinodiscus* spp. (0.23±0.32SD mm diameter) matched with the mouth gape dimension of *N lankesteri* as optimal prey size (Gerking, 2014). Both were relatively small compared to the mouth gape dimension of *N. lankesteri*. Therefore, the dimension of the prey items could support the fact that either diatom could be accidentally ingested or it might select directly on diatoms while the main prey item of *N. lankesteri* was copepod.

Teeth of *N. lankesteri* are unicuspid, found on the premaxilla (upper jaw) and the dentary (lower jaw). *Neostethus lankesteri* has a row of small teeth in the lower jaw, which are opposed to a row of larger teeth on the upper jaw. The premaxilla bears a series of larger teeth, which canine shaped located at edge of snout (Figure 3.5).

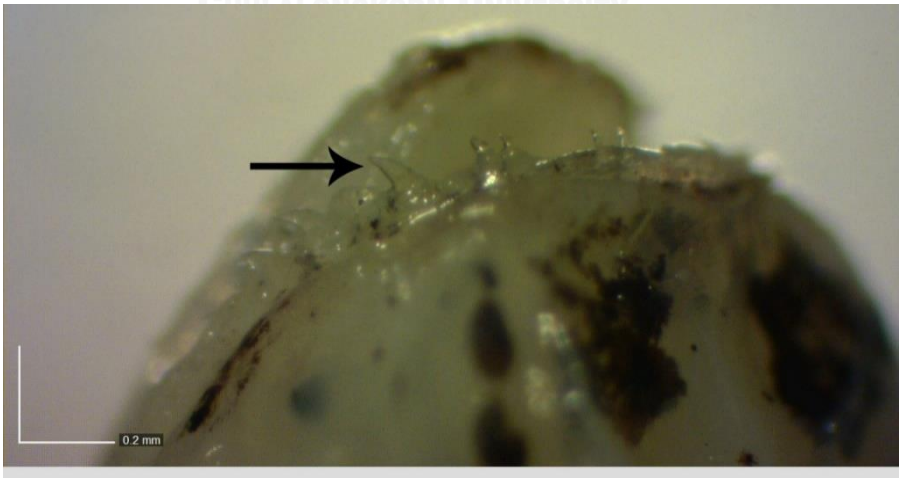

Figure 3.5 Premaxilla canine-liked teeth of *Nestethus lankesteri* (2.6 cm length) collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016. Arrowhead indicates canine-liked teeth.

It is similar to closely related species *N. bicornis*, Mok and Munro (1991) suggested that the shape of the teeth of *N. lankesteri* and *N. bicornis* related to feeding behavior which suggested that they fed on small animals. *Neostethus lankesteri* has short gill rakers varied from 15-18 slits. Short gill rakers related to pick up plankton rather than filter them as similar to *N. bicornis* (Mok & Munro, 1991) and *Scatophagus argus* (Wongchinawit, 2007). The nature of the short gill rakers indicated the probability that this fish can feed on small prey item i.e. copepods as similar to what was found in *N. bicornis* (Mok & Munro, 1991). Several teleosts have their unique pharyngeal teeth form/shape related to fish feeding guild. For example, the durophagus fish (fish that fed on hard-structure organisms, such as corals, shelled mollusks, or crabs) have heavier round and strong pharyngeal toothplates (e.g., *Pogonias cromis* exhibits large) robust molariform teeth; the example of durophagus fish is *Anisotremus surinamensis* which exhibits short densely packed conical teeth (Grubich, 2003). The non-durophagus fish (fish that fed on soft-preys species) tend to possess conical sharped form pharyngeal toothplates e.g., *Sciaenops ocellatus* and *A. virginicus* (Grubich, 2003). The pharyngeal teeth of *N. lankesteri* are numerous and canine-like shaped. The small teeth are arranged in a large number of parallel rows. It is similar to *N. bicornis* and *Oryzias javanicus* with well-developed pharyngeal teeth which suggested that they are carnivore (Mok & Munro, 1991). The pharyngeal teeth believed to act as a grinding mill (Tibbetts & Carseldine, 2005). The appearance of the pharyngeal teeth in *N. lankesteri* suggested that may function in mastication its prey before moved directly into the intestine portion.

The gross anatomy of *N. lankesteri* intestine consisted of three regions: the anterior intestine, middle intestine and posterior intestine under stereomicroscopic level (Figure 3.6B). The morphology of anterior intestine was the intestine bulb similar to previous reported (Mok & Munro, 1991). The middle intestine was a spiral shaped (Figure 3.6B(d)) before enter to posterior region.

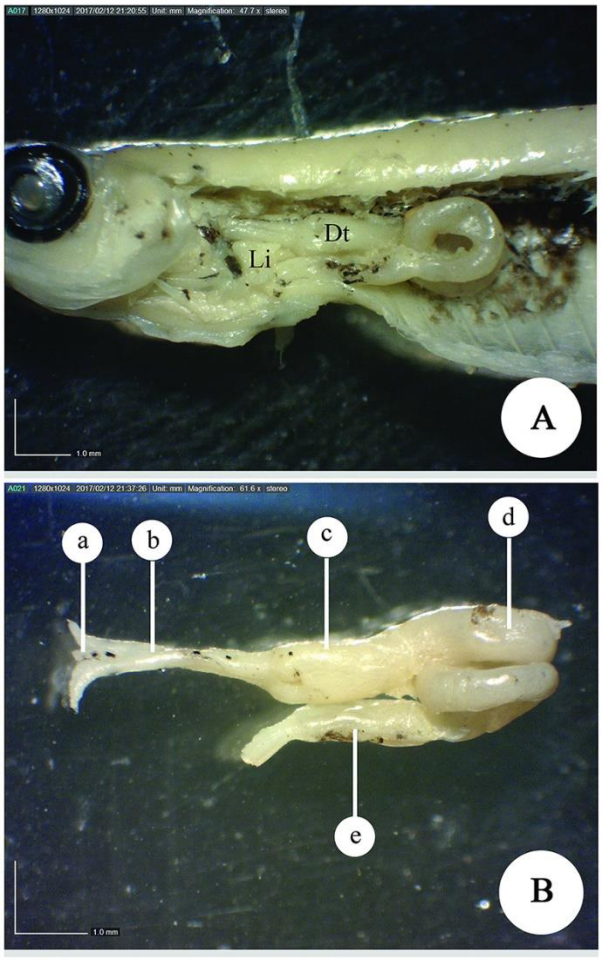

Figure 3.6 External morphology of digestive tract of *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016: **(A)** digestive tract orientation in the body cavity and **(B)** composition of digestive tract; a = pharyngeal toothplate, b = esophagus, c = anterior intestine, d = middle intestine, and e = posterior intestine.

The characteristic of the posterior intestine was a similar to anterior intestine but somewhat smaller than that region. This situation was similar to the previous investigation in *Phenacostethus smithi* and *Gulahallus mirabiis* (TeWinkel, 1939). The intestine coefficient of *N. lankesteri* in this study was 0.55, which was used to categorize species into different trophic levels or feeding type. According to previous observation which showed that low IC values suggested carnivore feeding guild (0.5-0.6 in *Sparus aurata* (Cataldi *et al.*, 1987) and 0.8 in *Glyptosternum maculatum* (Xiong *et al.*, 2011)), intermediate IC values suggested omnivorous feeding guild (2.0 *Ctenopharyngodon idellus* (Nie & Hong, 1963)) and high IC values (above 3.0 in

Puntius stoliczkanus (Senarat *et al.*, 2013)) suggested herbivores feeding guild. Therefore, the IC value of *N. lankesteri* is typically considered as carnivorous.

Histological and histochemical observations of digestive system

Oral cavity and pharynx

The oral cavity and pharynx was histologically observed with two layers including mucosa and submucosa; however, the muscularis was not observed (Figure 3.7A). The mucosa was lining with several layers of stratified polygonal epitheliums (Figure 3.7B). Each cell had thin nucleus and eosinopholic cytoplasm. Moreover, the large taste bud and mucous-secreting cells were observed among their epithelial cells (Figure 3.7B). The submucosal layer was identified composing of connective tissue under light microscopic level (Figure 3.7B). Another important organ, the tongue was centrally found in the mandible, which showed a mucosal thickening without muscular fibers. No teeth were exhibited on the tongue. The stratified epithelial layer of this organ was continuously seen from oral epithelium. This feature was not differed from those of other teleosts (*Decentrarchus labrax* (Abbate *et al.*, 2012)) and *Esox lucius* (Sadeghinezhad *et al.*, 2015). A thin layer of the loose connective tissue of the submucosa was detected. Note that this organ was supported by a large hyaline cartilage tissue (Figure 3.7C). The tongue plays a key roles in the ultimate acceptance or rejection of potential food items (Kruse & Stone, 1984). The pharyngeal teeth were clearly observed in the pharynx. The pharynx was lined by canine-liked shaped teeth (Figure 3.7D). It was histologically consisted of elongated immature and mature teeth within the stratified epithelium (Figure 3.7F). The taste bud and saccular mucous cells were also abundant and scattered among their epithelial cells (Figure 3.7H).

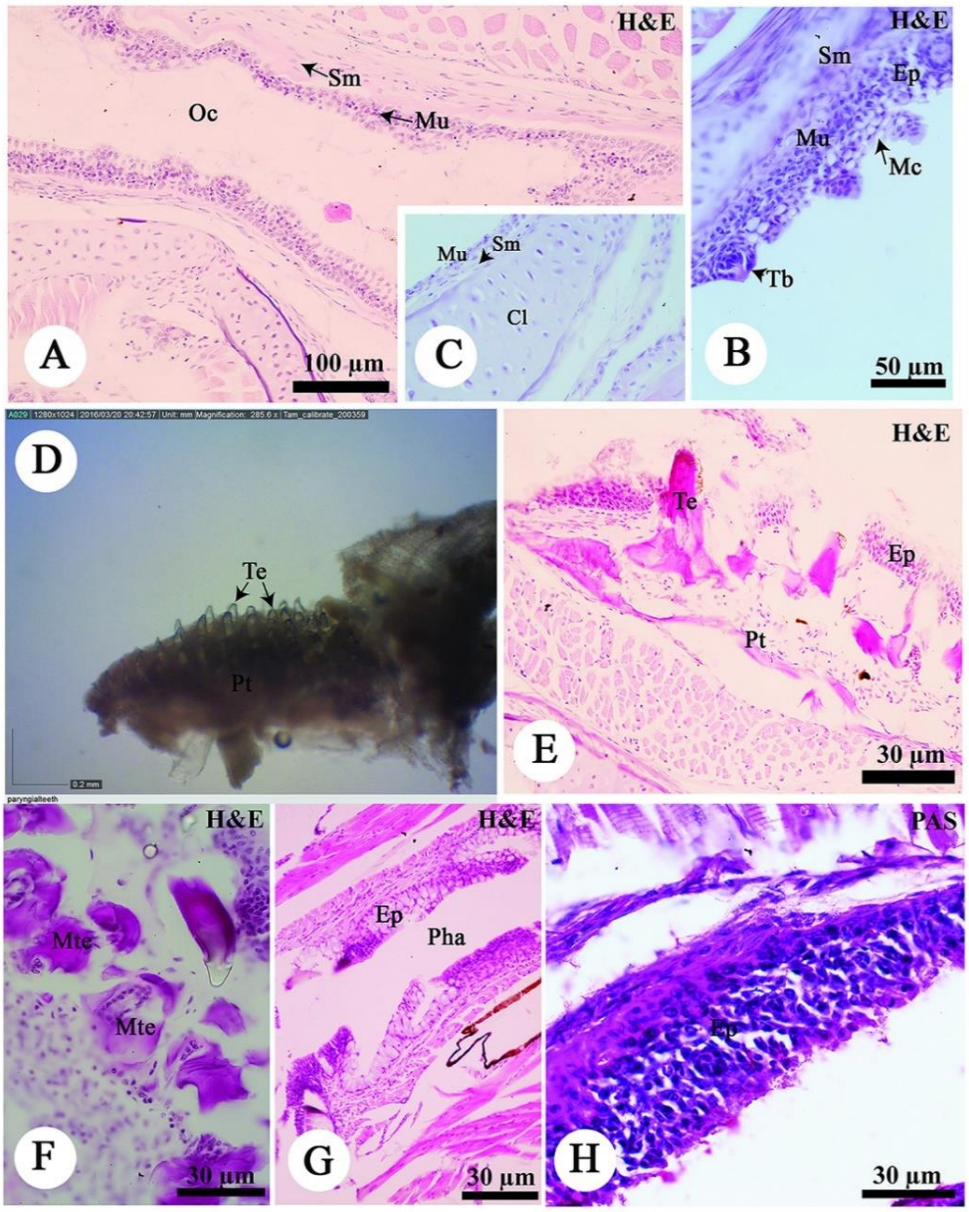

Figure 3.7 The oral cavity and pharynx histologically observation in *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016: **(A-C)** light micrograph of the oral cavity showing two layer of mucosa and submucosa, and **(D-H)** micrograph and transvers section of the pharyngeal toothplates. **Abbreviations:** Cl = cartilage tissue, Ep, epithelium, Mc = mucous-secreting cell, Mu = mucosa, Mte = mature teeth, Oc = oral cavity, Pha = pharynx, Pt = pharyngeal toothplate, Sm = submucosa, Tb = test bud, Te = teeth. **Staining:** H&E = hematoxylin and eosin, PAS = periodic acid schiff.

Esophagus

The esophagus was continuously observed from the pharynx (Figure 3.8A-B). The details of the esophageal wall were histologically composed of four layers including mucosa, submucosa, muscularis and serosa, respectively. However, the serosa was difficult to identify (Figure 3.8C). The longitudinal fold of the mucosal layer was lined by a protective simple cuboidal epithelium. Epithelial feature with similar pattern was reported in *Raja clavata* (Holmgren & Nilsson, 1999). Several mucous cells among the epithelial cells were negatively stained with H&E method (Figure 3.8D), but they were positively reacted with PAS method, indicating the presence the glycoprotein (Figure 3.8E). It is assumed that the glycoprotein helps lubricate the food substance during the transferring process to the esophageal and intestinal junction (Cataldi *et al.*, 1987; Grau *et al.*, 1992; Harder, 1975). A few layer of the lamina propria layer containing the loose connective tissue and submucosa was not easily identified under light microscopic level (Figure 3.8D). No esophageal gland was observed. The prominent of the muscularis was found and constituted in two layers (inner circular and outer longitudinal sub-layers) (Figure 3.8D).

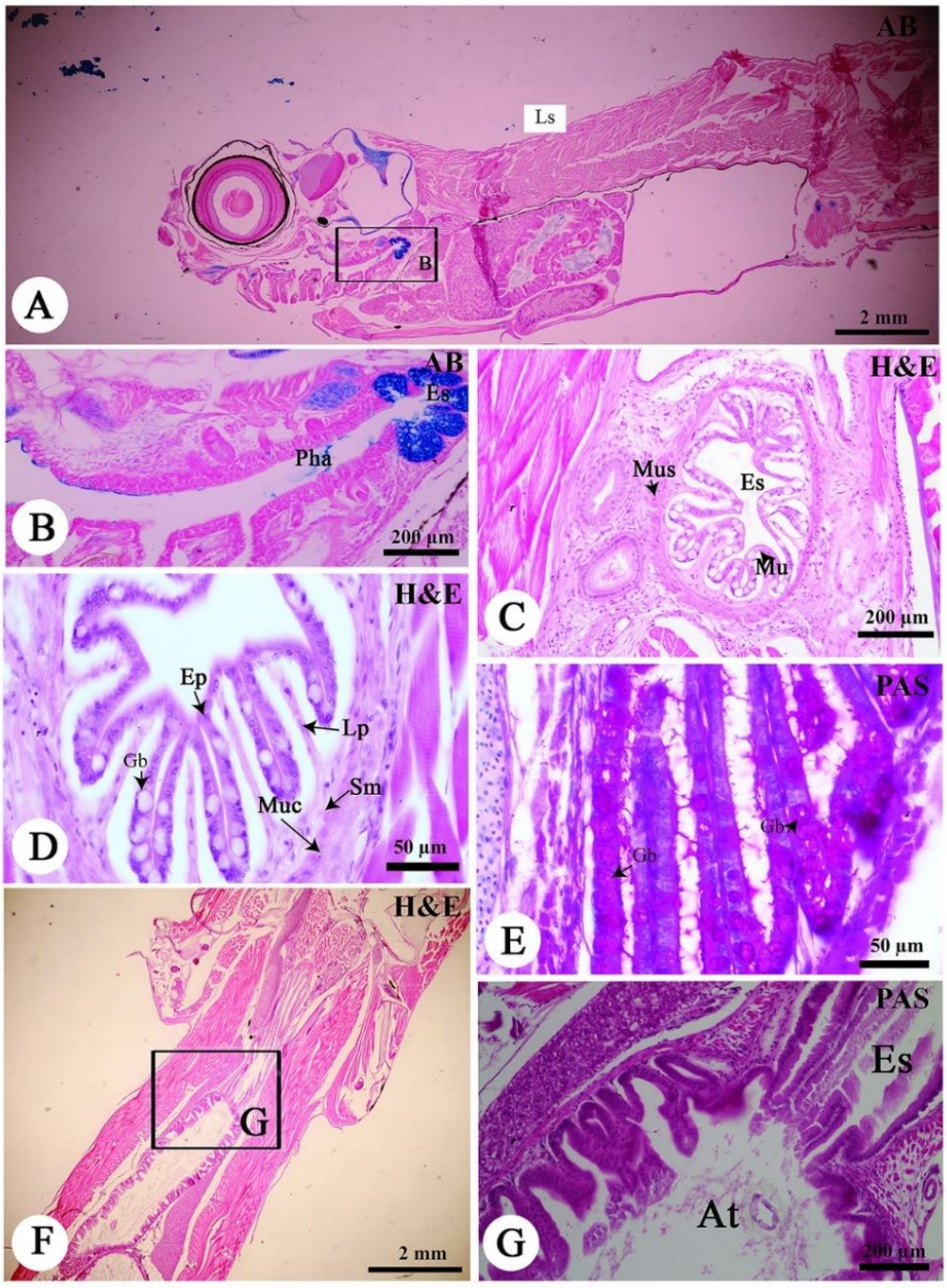

Figure 3.8 Light photomicrographs of esophagus region in *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016: **(A-B)** conjunction between pharynx and esophagus, **(C-E)** histological feature of esophagus showing several mucous cells in epithelial layer, and **(F-G)** the junction between esophagus and intestine region called esophageal and intestinal junction. **Abbreviations:** At, anterior intestine, Es = esophagus, Muc = mucous cell, Lp = laminar propria, Ls = lateral side, Mus = muscularis. **Staining:** AB = alcian blue and PAS = periodic acid schiff.

Intestine

High magnification showed the junction between esophagus and intestine (Figure 3.8F-G). The histological feature of the esophageal lining changed from simple high cuboidal epithelium into simple columnar epithelium (Table 3.2). It was possible that the structural changed would be an increased function related to the breakdown of ingested food before entering the intestine. In this present study, the intestinal region could be classified according to the localization and histological structure into three regions including anterior intestine, middle intestine and posterior intestine (Figure 3.9).

Table 3.2 Major histologically variation among pharynx, esophagus and intestine of *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center.

Organs	Epithelial Mucosa	Mucous cell	Submucosa	Muscularis (inner circular and outer longitudinal sub-layers)
Pharynx	SPE		present	
Esophagus	SCE	present	present	present
Anterior intestine	SCE	present	present	present
Middle intestine	SCE	present	present	present
Posterior intestine	SCE	present	present	present

Note: SCE; simple columna epithelium, SPE; stratified polygonal epitheliums and SSE; simple squamous epithelium.

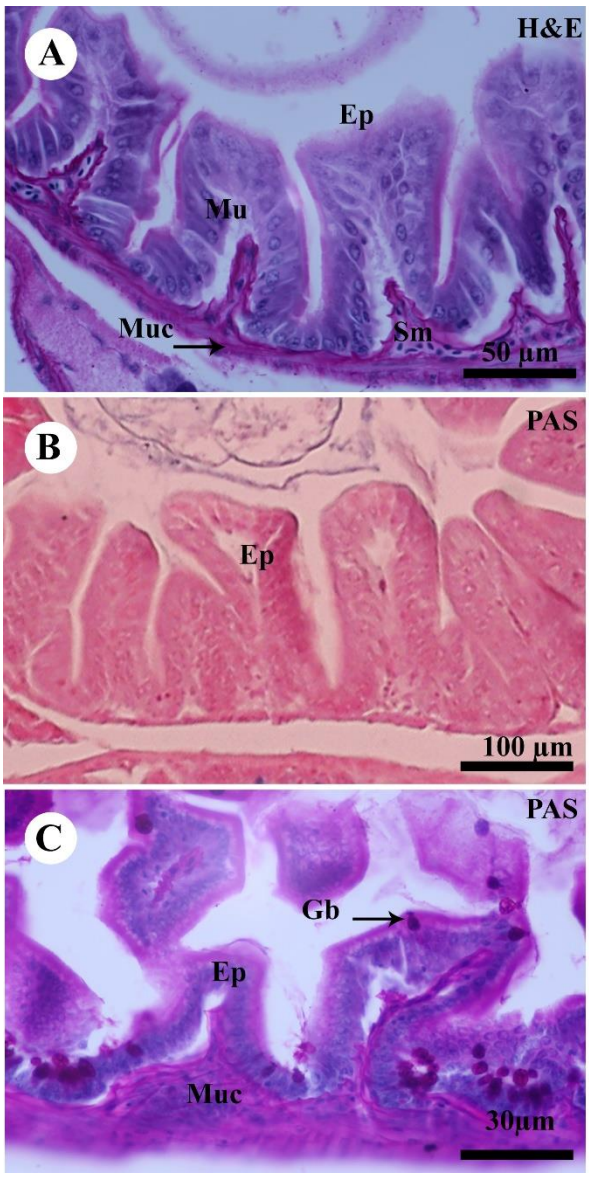

Figure 3.9 Transvers sections of intestine regions of *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016 showing longitudinal folds length different among three regions: **(A)** anterior intestine, **(B)** middle intestine, and **(C)** posterior intestine. **Abbreviation:** Ep = epithelium, Gb = goblet cell (arrowhead), Mu = mucosa, Mus = mucous cell and Sm = submucosa.

In the anterior intestine, the prominent of the longitudinal fold in the mucosal layer was strongly identified composing of two sub-layers: epithelial and lamina propia sub-layers (Figure 3.9A). The epithelial layer was covered by a simple columnar epithelium. Loose connective tissue was the most common tissue of the lamina propria, while the identification of the muscularis mucosae and submucosa were difficult under

light microscopic level. Muscularis was obviously seen in this species and composed of two layers; inner circular and outer longitudinal sub-layers.

The middle intestine and posterior intestine histological structures were commonly similar to anterior intestine (Figure 3.9B-C). However, longitudinal fold were significantly different ($P<0.0001$) among anterior (79.06±15.3SD μm), middle (130.85±28.8SD μm), and posterior intestine (43.09±18.6SD μm). Furthermore, several goblet cells or mucus-secreting cell were also seen (middle intestine and posterior intestine) and positively reacted with PAS method (Figure 3.9C), indicating the presence of glycoprotein. It is similar to others teleosts such as *Belone belone* (Bočina *et al.*, 2017) and *Seriola dumerili* (Grau *et al.*, 1992). Several goblet cells represented the function of the cell in nutrients absorption, lubrication and protection of epithelial layer (Bočina *et al.*, 2017; Oliveira Ribeiro & Fanta, 2000). However, the goblet cells in the last portion of intestine suggested for increased mucous production to lubricate food particles, defecation, and also for protecting the epithelial layer rather than function in absorptive ability (Murray *et al.*, 1994; Oliveira Ribeiro & Fanta, 2000; Purushothaman *et al.*, 2016).

Since primary functions of the stomach are to store food and digest protein associated to gastric gland which produce digestive enzymes i.e. pepsinogen (Day *et al.*, 2011). Some fishes had stomachless. *Neostethus lankesteri* did not have a well-defined stomach. This is similarly found in *Belone belone* (Bočina *et al.*, 2017). The intestine of stomachless carnivorous fish i.e. Belonidae had been suggested to produce enzymes trypsin and aminopeptidase locally in the posterior portion of intestine for protein digestion. This type of stomach plays similar role to the pepsinogen produced stomach (Day *et al.*, 2011). Moreover, stomachless fishes such as beloniforms tend to have large pharyngeal teeth that act as a grinding mill (Tibbetts & Carseldine, 2005), similar to the *N. lankesteri* in this study. The appearance of large pharyngeal teeth may act as a grinder for its prey before moving the prey directly into the intestine portion where nutrients absorption occurs. Overall characterization of digestive tract included mouth, pharyngeal teeth, gill racker, esophagus, and intestine of *N. lankesteri* implied that these characters evolutionary adapted to consume and absorb nutrients of zooplankton, and support the general idea that the priapiumfish *N. lankesteri* is zooplankton feeder.

Liver and Pancreatic tissue

Overall histologically features of liver and pancreatic of *N. lankesteri* were mostly similar to those of other carnivorous fishes (Bočina *et al.*, 2017; Day *et al.*, 2011). All liver sections revealed that the histological structures in this species contained the cords of hepatocytes (Figures 3.10A-B). Each cord was separated by hepatic sinusoid which contained several erythrocytes (Figure 3.10A). Each hepatocyte was polygonal shape, which within the hepatocytes was positively reacted with PAS method. Hence, this study confirmed that it produced the glycoprotein substrates (Figure 3.10C). The pancreatic tissue was located and scattered near the intestine (Figure 3.10D). High magnification showed that it composed of two tissues including the endocrine pancreas and islet of Langerhans. The endocrine pancreas was composed of a cluster of the pyramidal pancreatic cells. Each cell was contained in several large eosinophilic zymogen granules.

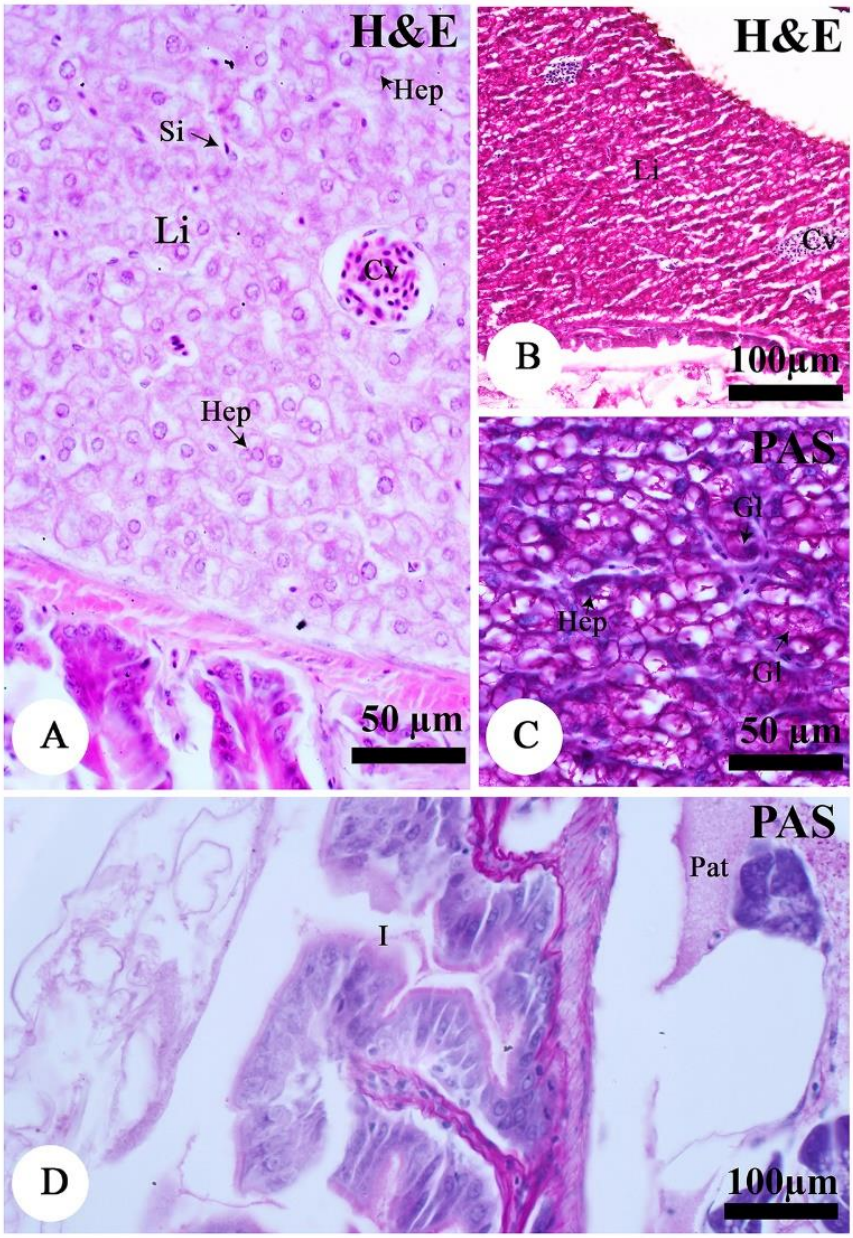

Figure 3.10 Transvers section of liver and pancreas in *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016: **(A-C)** light micrograph of liver and **(D)** location of pancreas. **Abbreviations:** Cv = central vessel, Gl = glycoprotein, Hep = hepatocyte, I = intestine, Li = liver, Pat = pancreatic cell, Si = sinusoid.

Conclusion

In conclusion, this study provided ecological and histological evaluations of the gut content and morpho-histological characteristics of the digestive system in the priapiumfish *N. lankesteri* from Pranburi River estuary and Sirinaj Rajini Mangrove Ecosystem Learning Center. Based on IRI analysis, the food items of the *Neostethus lankesteri* can be classified into four groups: diatoms, algae, dinoflagellate and zooplankton. In this study, *Neostethus lankesteri* showed variety of diets, the quantity of the prey items in the gut content from the Southwest monsoon were not significant different from the prey items found in the Northeast monsoon. On the other hand, the preys items in gut content of this fish from Pranburi River estuary were significant different (P=0.00) from those that were from SRMELC. The gut content of *N. lankesteri* from Pranburi River estuary from both seasons showed that the major component of the diet was diatoms. In contrast, the diet component of *N. lankesteri* from SRMELC showed that the major component was zooplankton from both seasons. Based on the gut content it can be assumed that either the diatom in the gut content of *N. lankesteri* might be accidental ingested or *N. lankesteri* select directly on diatoms. However, the morpho-histology of the gut including canine teeth on lips of mouth, short gill rakers, and short intestine coefficient implied that *N. lankesteri* is a carnivorous fish and generalist feeder on zooplankton.

CHAPTER 4

REPRODUCTIVE PATTERN AND OVARIAN DIFFERENTIATION OF *Neostethus lankesteri* Regan, 1916 FROM PRANBURI RIVER ESTUARY, THAILAND

Introduction

The reproductive pattern or feature in fish has been wildly reported, which is important to accurately determine gonadal tissue and gametogenesis in order to fisheries management (Marques et al., 2000). Using the light microscopic level, the classifications of gonadal development and gametogenesis in a variety of teleost have been described (Al-Daham & Bhatti, 1979; Mayer *et al.*, 1988). This included *Fundulus heteroclitus* (Kuchnow & Scott, 1977), *Oryzias latipes* (Wallace & Selman, 1981), *Dicentrachus labrax* (Mayer *et al.*, 1988) and *C. undecimalis* (Neidig *et al.*, 2000), *T. orientalis* (Chen *et al.*, 2006) and *Serra Spanish* (Chellappa *et al.*, 2010a). The oocytes were generally produced within the ovigerous fold, which has been divided into four stages according to their histological structures: oogonia, immature oocyte, maturing oocyte and mature oocyte. Moreover, the oogenic stage is classified by the uptake of vitellogenin or yolk protein: previtellogenic, vitellogenic and postvitellogenic or mature stage (Gupta, 1975). Gupta (1975) and Uribe *et al.* (2012) also purposed the division of oocyte stages as follows: oogonia proliferation stages, chromatin-nucleolus stage, primary growth stage and secondary growth stage. In male fish, there have been many studies about the testicular structure and spermatogenesis such as *Scomberomorus tritor* and *Cybium tritor* (Mattei, 1991), *S. japonicas* (Hara & Okiyama, 1998; Mattei, 1991), *Scomber australasicus* (Hara & Okiyama, 1998), and *Thunnus thynnus* and *Euthynnus alletteratus* (Abascal & Medina, 2005). The testicular structure contained numerous convoluted seminiferous lobules, which were classified into two compartments: interstitial compartment and germinal compartment. The various developmental stage of the spermatogenic cells were produced in the germinal compartment. It was generally divided into three distinct phase including spermatogonial phase, spermatocyte phase and spermiogenetic phase (Schulz *et al.*, 2010).

Although, there have been reports concerning the gametogenic stages in Family Phallostethidae (Grier *et al.*, 1980; Grier & Parenti, 1994; Mok & Munro, 1997; Parenti & Grier, 2004), none covered in *Neostethus lankesteri* Regan, 1916. This fish is reported as a dominant species and is part of important estuarine fisheries in Pranburi River estuary, Thailand (Paphavasit *et al.*, 2014). They were previously believed to play an important role in linking the secondary consumer under the ecological system. Additionally, the unique characterization on Family Phallostethidae showed the secondary reproductive organ called "the priapium organ". This organ is modified from the pelvic fin. The important role of the priapium organ concerns with the transfer of spermatozoa from the male to the female reproductive tract (Grier & Parenti, 1994). In this study, mature reproductive pattern and ovarian differentiation of *N. lankesteri*, using histological and histochemical approaches was determined. A better comprehension of the gametogenic process of this species provides important information before beginning to assess the spawning season, health status and systematic/ phylogenetic characters.

Materials and methods

Fish sampling

Two hundred and thirty healthy *N. lankesteri* (1.5 to 2.4 cm (SL)) were collected by larval fish otter trawl from the Pranburi River estuary, Prachuap Khiri Khan Province (12°24'21.6 " N, 099°58'37.1" E and 12°23'53.77" N, 099°58'55.98" E) in March 2015 and then euthanized by rapidly cooling shock. From the 230 processed specimens, only 53 specimens (1.5 to 2.4 cm SL) provide adequate microstructure detail for the gametogenesis study. The experimental protocol was approved by the Animal Care and Use Committee of Faculty of Science, Chulalongkorn University (Protocol Review No. 1523005).

Reproductive pattern

To assess the reproductive pattern, 2.4 cm (SL) were collected fish of both sexes (n=10). The samples were routinely processed according to standard histological techniques (Presnell & Schreibman, 1997; Suvarna *et al.*, 2013). All paraffin blocks

were sectioned at 4-5 µm thickness and stained with hematoxylin–eosin (H&E), Masson's Trichrome (MT), Alcian blue (AB) and Periodic Acid Schiff (PAS) (Presnell & Schreibman, 1997; Suvarna *et al.*, 2013). The gonadal features were viewed, photographed and measured under a light microscope (Leica digital 750). Ninety to one hundred oocytes were randomly measured from three histological sections. Each section was randomized in three areas (Brown-Peterson *et al.*, 2011; Dietrich & Krieger, 2009). Schematic diagram of the ovarian tissue was done with Adobe illustration CS6.

Ovarian differentiation

Five individuals for each size of females (total specimens = 45 samples) were collected for the study of ovarian differentiation and ovarian structure. They were processed in a similar manner using a standard histological techniques. The feature of the ovarian differentiation was observed and photographed under a light microscope (Leica digital 750). Schematic diagram of the ovarian differentiation was done with Adobe illustration CS6.

Priapium organ

To study the structure of the priapium organ in male *N. lankesteri* (n = 3 with 2.4 cm of the standard length), it was observed cleared and stained specimens (Potthoff *et al.*, 1986). As to detail, the priapiums were fixed in 10% neutral buffered formalin (NBF) and immersed in 2% potassium hydroxide (KOH) with 3% hydrogen peroxide (H_2O_2). They were then stained with alcian blue and alizarin red stain and finally replaced in glycerin. All samples were observed in both localization and histology and photographed with Olympus TG-2.

Results and discussion

Female reproductive pattern of *Neostethus lankesteri*

Ovarian structure and oogenic steps

The morphology of the mature ovaries of *N. lankesteri* were observed. They had a single ovary with a creamy color (Figures 4.1A-C). It is longitudinally suspended by

mesovarium in the abdominal cavity. Overall histological structure and schematic diagram of the mature ovarian tissue in both longitudinal and cross sections composed of the ovarian tissue and oviduct. The ovarian tissue contained several developmental stages of oocyte (Figures 4.1 and 4.2). However, the ovigerous fold was difficult to classify. It was also considered as an asynchronous developmental type (Figures 4.1B and 4.1D). This is similar to *Hyporhamphus regularis ardelio* (Nuttall *et al.*, 2012) and other fishes (Chellappa *et al.*, 2010b). This characterization implied that *N. lankesteri* may be considered as a protracted spawner which could have multiple spawning seasons. The oviduct of this fish positioned into two regions based on the localization and histological structure; primary and secondary oviducts (Figures 4.1E-G and 4.1a). The primary oviduct was observed in the middle area (dorso-ventral axis) of the ovary (Figures 4.1B and 4.1D), whereas the feature of the secondary oviduct was jointed with the primary oviduct in the posterior part before opening separately into the cloaca (Figure 4.1a). This is in contrast to the salmonids which lack of the oviductal structure (Gupta, 1975; Redding & Patino, 2000). Histologically, all oviductal regions were composed of tunica mucosa, tunica muscularis and tunica serosa. A slight irregular folds of the tunica mucosa in the primary oviduct were clearly lined by simple columnar epithelium (Figures 4.1E-F). The mucous secretory cells were spherical shape and rarely seen (Figure 4.1E). Previous observation reported that the role of the mucous secretory cell related to the level of the chemical composition and implied to support during gamete transport (Mamillan, 2007). The primary oviduct was also surrounded by a thin layer of the muscularis (Figure 4.1E). The histological structure of the secondary oviduct was differed from the primary oviduct. As the primary oviduct was covered by a simple columnar epithelium. Two muscularis layers including circular and longitudinal muscularis layers was obviously identified in this region (Figure 4.1G). It was possible that tunica muscularis could related to the movement of the oviduct structure under the process of the ovulation. In the present study, five phases of the various developmental stage of oogenic stage in *N. lankesteri* were distinctly classified according to staining of sections, homogeneity and histological features of sex cells into oogonia proliferation, primary growth phase, secondary growth phase, post-ovulatory phase and atretic follicle phase. This is similarly seen in several hemiramphids such as

He. brasiliensis, *He. balao* (McBride & Thurman, 2003) and *H. melanochir* (Ling, 1958).

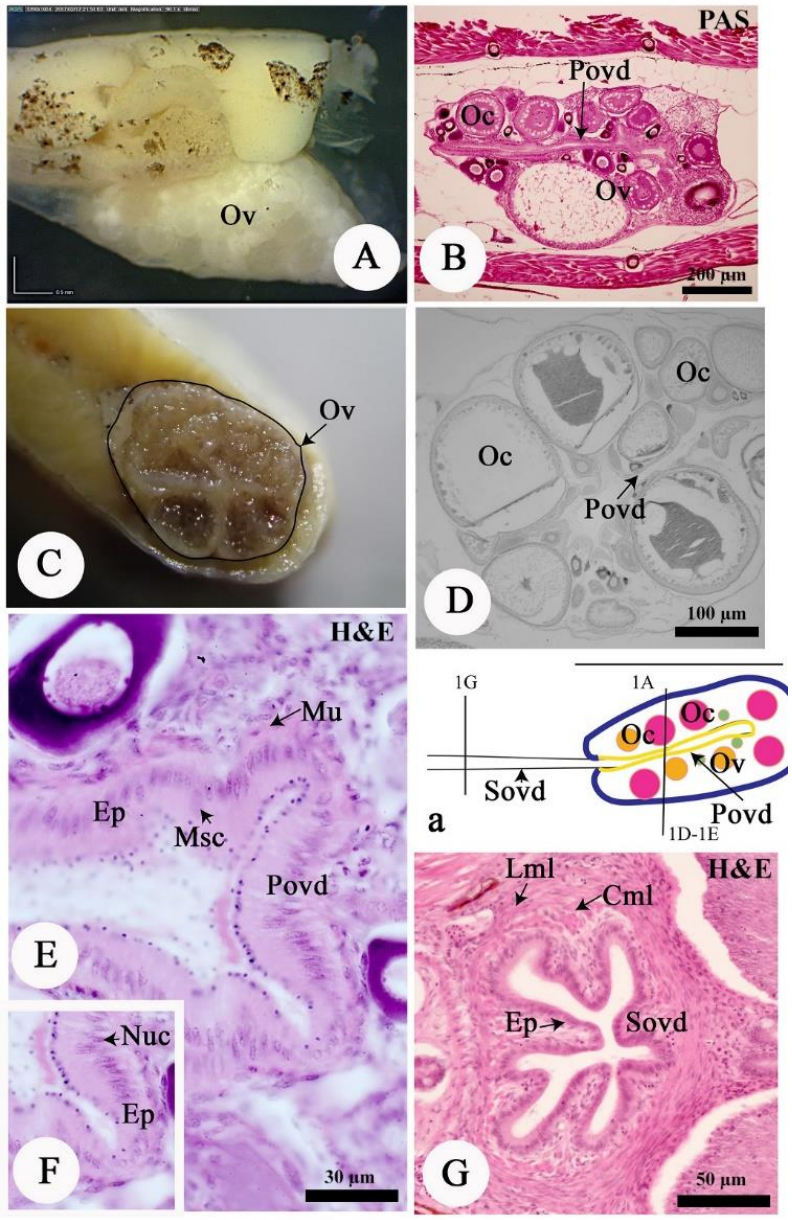

Figure 4.1 Morphology **(A, C)** and light photomicrograph **(B, D, E-G)** of the ovarian structure (Ov) with various developing oocytes (Oc) and oviducts. A – B longitudinal section & C- D cross section of the ovary in *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center. **Abbreviations:** Cml = circular muscularis layer, Ep = epithelium, Lml = longitudinal muscularis layer, Mu = muscularis, Msc = mucous cell, Nuc = nucleus, Povd = primary oviduct, Sovd = secondary oviduct. **Staining:** H&E = hematoxylin and eosin, PAS = periodic acid schiff.

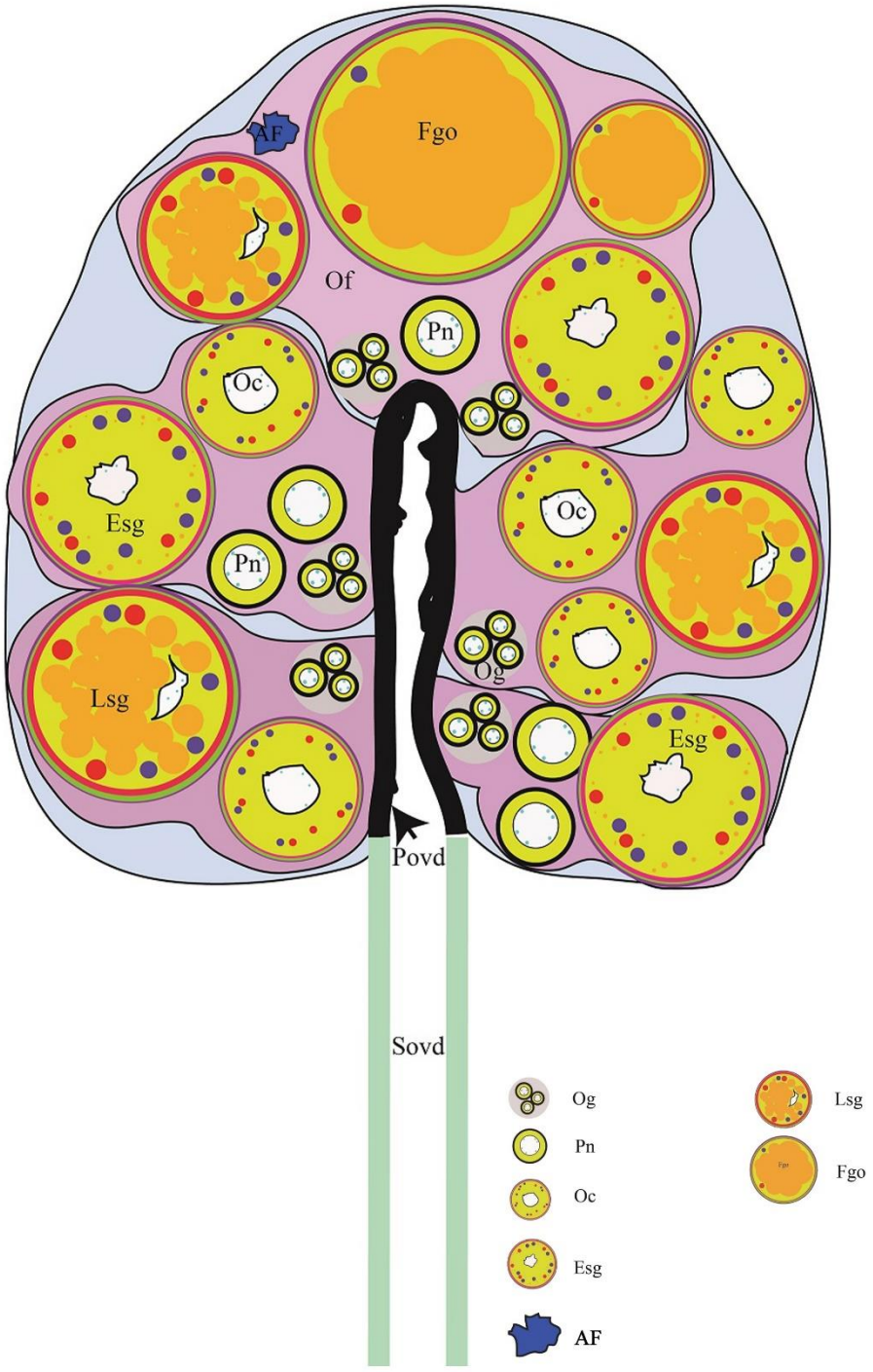

Figure 4.2 Schematic diagram showing the ovarian structure and oogenesis in *Neostethus lankesteri*. **Abbreviations:** Of = ovigerous fold, Povd = primary oviduct, Sovd = secondary oviduct.

Oogonia proliferation

The oogonium was the smallest cell size among the oogenic cells. It was located in the germinal epithelium (Figure 4.3A). It was an oval-rounded cell. A spherical nucleus shape contained a prominent nucleolus and slight eosinophilic cytoplasm (H&E method) (Figure 4.3A). The oogonium was formed within clusters or oogonial cysts inside the ovigerous fold. The oogonium was surrounded by a squamous-shaped prefollicular cells (Figure 4.3A).

Primary growth phase

This phase consisted of two sub-phases of oocyte in the mature ovaries including perinucleolar (Pn) and oil droplets and cortical alveolar stage (Oc)

The size of Pn was about 20 µm in diameter in *N. lankesteri*. The central nucleus was large with a spherical shape (Figure 4.3A-B). Multiple nucleoli with varying diameters were positioned adjacent to the nuclear membrane (Figure 4.3A-B). The first appearance of Balbiani's bodies were observed in this phase (Figure 4.3A), as similarly found in other fishes (Wallace & Selman, 1981). The function of Balbiani's body was unclear, but it was believed to involved with the primary oogenic process because its structure have more complex organelles functions (rich in nucleic acid, RNA and proteins, mitochondria, centrioles, Golgi bodies, and endoplasmic reticulum) (Hamaguchi, 1993; Selman & Wallace, 1989). A strong basophilic staining of the ooplasm was also seen. The presence of ooplasmic basophilia during primary growth stage is the characteristic of a period of intense RNA synthesis together with ribosome production to support the oocytes development (Wallace & Selman, 1990).

The Oc reached 71 µm in diameter in *N. lankesteri*. The nucleus increased in size during this stage (Figure 4.3C). The nucleoli at the periphery of the slight folding nucleus were increasingly seen in number (Figure 4.3C). The slight basophilic ooplasm were contained in several inclusions. Such the oil droplets and cortical alveoli were first dispersed at the periphery of the oocyte (based on H&E method, Figure 4.3C). Empty vacuoles of the oil droplet were negatively stained in preparations stained with H&E and PAS methods (Figure 4.3C-D). The function of this inclusion concerned with energy sources for embryo development (Wiegand, 1996). The cortical alveoli were

spherical in shape at the periphery of the ooplasm, showing varying size of diameter. This inclusion was positively reacted with PAS and AB stains, indicating the presence of the glycoprotein and mucopolysaccharide (Figure 4.3D). The role and function of the cortical alveoli concerned with physiological roles especially the prevention of polyspermy after ovulation (Nagahama, 1983). At this stage, the follicular complex was initially well defined and was clearly consisted of three layers including zona pellucida, granulosa cell and theca cell (Figure 4.3C-D). A thin layer of zona pellucida was clearly seen as a cellular acidophilic structure and was found between the oocyte and follicular cells (Figure 4.3C-D). The acidophilic fibrils were locally seen between single layers of granulose cells and theca cells in various positions (Figure 4.3C-D). Similar fibril composition occurred in the fish oocyte (Dumont & Brummet, 1980; Patiño *et al.*, 2003). Previous studies provided conflicting data about the function of the fibril. However, some investigators suggested that it may help to prevent water loss during low tide (Dumont & Brummet, 1980).

Secondary growth phase

This phase could be found in three stages of oocyte in the mature ovaries including early secondary growth step (Esg), late secondary growth step (Lsg) and full-grown oocyte step (Fgo).

The Esg had increased in size the reaching diameters of 97 µm. The histological structure of the nucleus was obviously folded (Figure 4.3E). The major characterization showed that the small yolk granules were observed in both spherical and deeply acidophilic stain in the ooplasm (Figure 4.3E). The Esg began to accumulate at the oocyte periphery, as previously found in most fishes (Chen *et al.*, 2006). This inclusion were the material stored during the secondary growth stage (Wallace & Selman, 1990) and generally related to support the embryonic development as major source of the nutrition and metabolic activities in most fish species (Chen *et al.*, 2006). The oil droplets and cortical alveoli remained, but these inclusions progressively increased in both number and size (Figure 4.3E). The zona pellucida was well-differentiated, distinctly striated and increased further in thickness (Figure 4.3E). The increasing number of acidophilic fibrils was well defined (Figure 4.3E). The granulosa cell remained as a single layer, however its shape changed from squamous to low columnar

epithelium (Figure 4.3E). A layer of theca cells was also similarly exhibited at that prior stages.

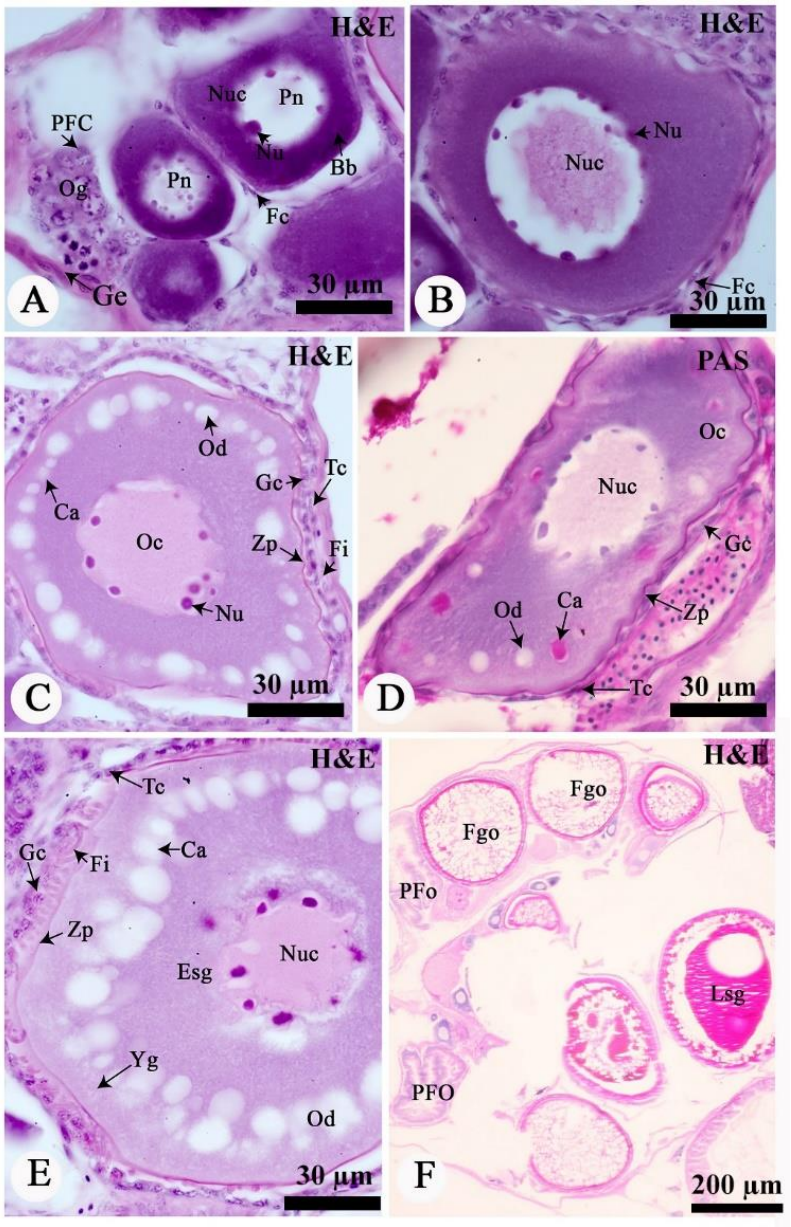

Figure 4.3 Light photomicrograph of various developing oocytes in *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center. **Abbreviations:** Bb = Balbiani's body, Ca = cortical alveoli, Esg = early secondary growth step, Fc = follicular cell, Fi = fibril, Gc = germinal epithelium, Fgo = Full-grown oocyte step, Gc = granulosa cell, Lsg = late secondary growth step, Nu = nucleolus, Nuc = nucleus, Oc = oil droplet and cortical alveolar stage, PFC = pre-follicular cell, Pn = perinuceolar stage, POF = post-ovulatory follicle, Tc = theca cell, Yg = yolk granule, Zp = zona pellucida.

The Lsg was increased its size up to 149 mm in diameter because the oocyte contained a lot of yolk granules and progressively increased in number and size (Figure 4.3F). It was similarly seen in *Hemiramphus brasiliensis* and *He. balao* (McBride & Thurman, 2003). Some areas of ooplasm initially revealed the yolk granules fusion (Figure 4.4A and 4.4D). The oil droplets and cortical alveoli were progressively observed in size and distributed throughout the ooplasm (Figure 4.4D). The zona pellucida with striated structure was observed and clearly divided into a thin external layer and thick internal layer. The layers of the granulosa and theca cells were not different from the previous stage (Figure 4.4B).

The Fgo was observed at the end of the oogenic process and considered as the largest oocytes, reaching diameter of 218 μm in diameter. No nucleus was observed. The yolk globules were fused completely and negatively stained with H&E stain (Figure 4.4C) with unclear reason.

Post-ovulatory phase

The presence of the postovulatory follicles (POF) was observed in *N. lankesteri*. It composed of the follicular layers, referring to the layers of the granulosa and theca cell. This was the remaining structure in the ovary of fish after the release of the ovum during spawning (Figure 4.4F). The fact that all ovarian developmental stages and the postovulatory stage were observed in this study suggested that *N. lankesteri* spent their entire life cycle in the Estuary which could infer that they are true estuarine species.

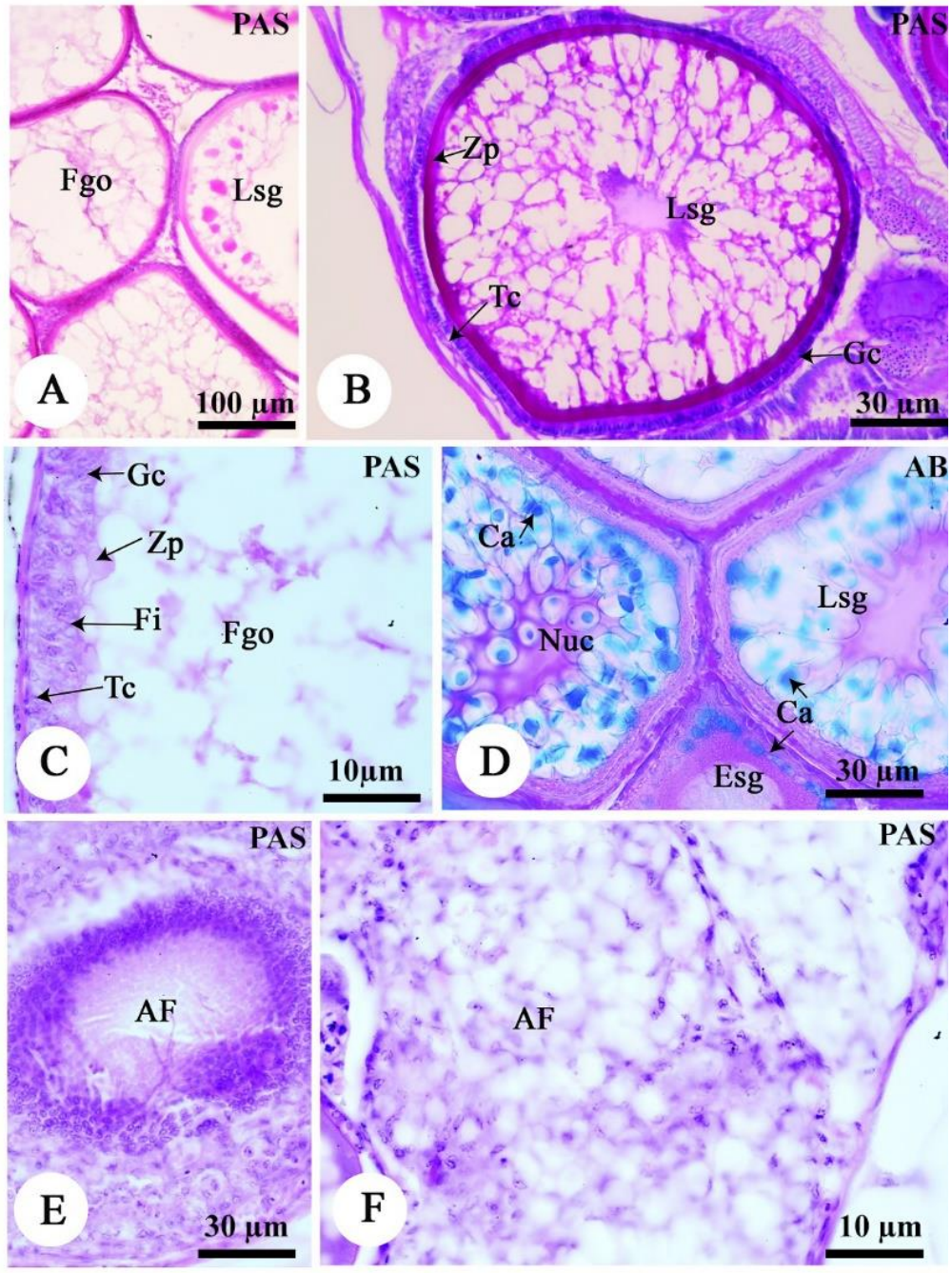

Figure 4.4 Light photomicrograph of various developing oocytes in *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center. **Abbreviations:** AF = atretic follicle, Ca = cortical alveoli, Esg = early secondary growth step, Fi = fibril, Gc = germinal epithelium, Fgo = Full-grown oocyte step, Gc = granulosa cell, Lsg = late secondary growth step, Nuc = nucleus, Oc = oil droplet and cortical alveolar stage, Tc = theca cell, and Zp = zona pellucida. **Staining:** AB = alcian blue and PAS = periodic acid schiff.

Atretic follicle phase

Atretic follicle was detected in *N. lankesteri* throughout the study (Figures 4.4E-F). The characterization of this stage was irregular in shape, convoluted layer of granulosa cell formed inside the atretic follicle cell. Previous observation revealed that the occurrence of the atretic follicle in teleosts related to stresses and other environmental conditions e.g., insufficient nutrition, physical factors, hormones and chemical factors (Dietrich & Krieger, 2009).

Male reproductive pattern of *Neostethus lankesteri*

Testicular structure and spermatogenic steps

The morphology of the mature testicular structure was a single and unpaired organ (Figure 4.5A-B) which was elongated and suspended from the dorsal peritoneum. Based on the longitudinal section, the testicular structure was surrounded by tunica albuginea (Figure 4.5C), which was composed of prominent connective tissue, blood vessels and a few layers of smooth muscle. The testicular parenchyma was composed of two regions, testicular structure and a well-developed efferent duct system (Figure 4.5C-D). It is basically similar to *Gulaphallus mirabilis* (Grier *et al.*, 1980) and *Phenacostethus smithi* (Grier & Parenti, 1994; Munro & Mok, 1990).

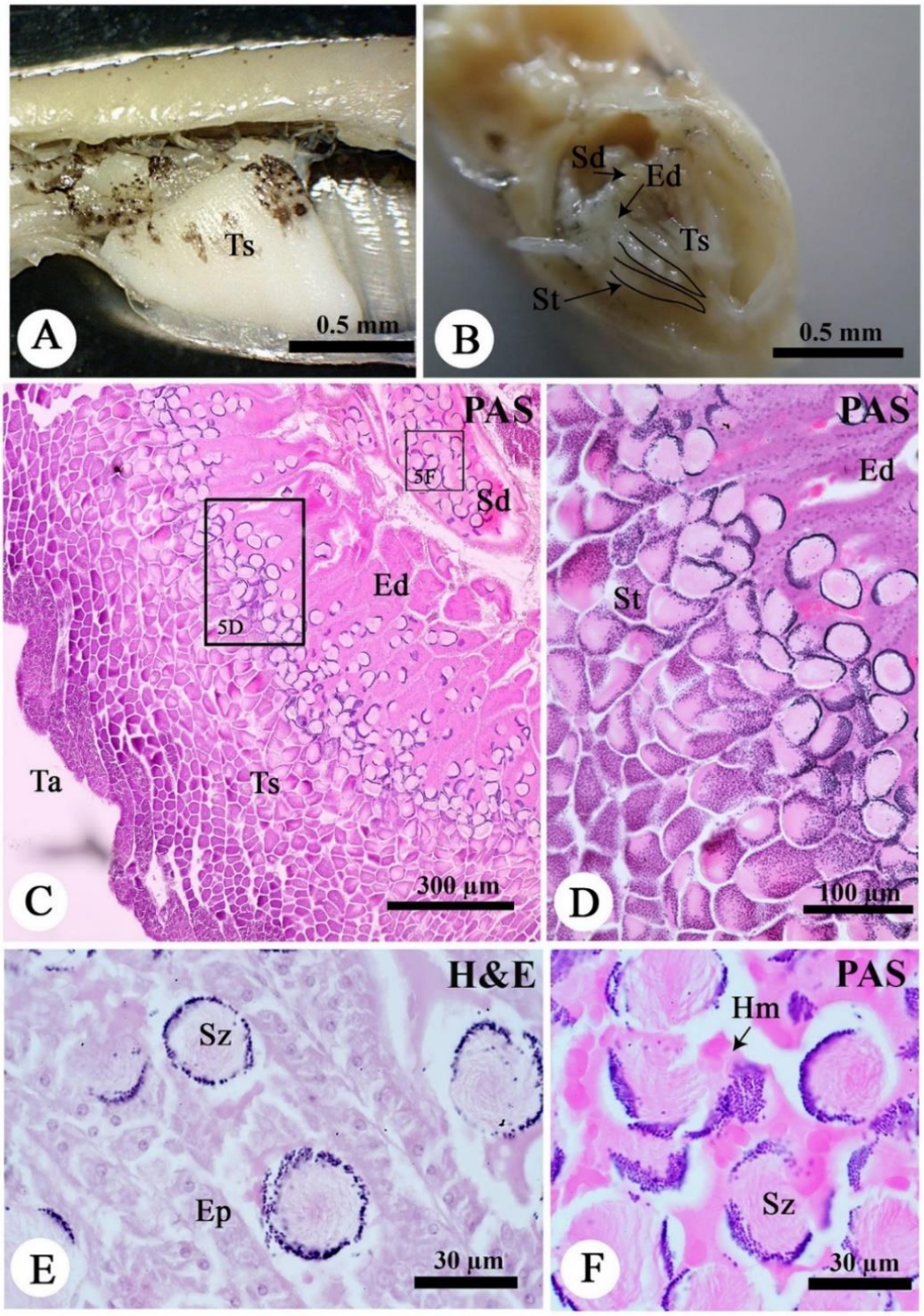

Figure 4.5 Morphology and light microscope of the testicular structure (Ts) and testicular ducts in *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center. **Abbreviations:** Ed = efferent duct, Hm = a homogenous eosinophilic matrix, Sd = spermatic duct, St = seminiferous tubule, Sz = spermatozoa, Ta = tunica albuginea.

Several seminiferous tubule was identified in the testicular structure and then each tubule was jointed and became the efferent duct and spermatic duct at the posterior region (Figure 4.5B-D). The efferent duct containing the mature spermatozoa was lined by a simple columnar epithelium (Figure 4.5E). A thin layer of the muscularis was also found. The covering epithelium of the spermatic duct was similarly in the efferent duct, however, it was embedded in a homogenous eosinophilic matrix (H&E method) (Figure 4.5F). This substance may be secreted from the epithelium to support the sperm maturation. The function of two ducts is carrying mature sperm towards the vas deferens during spermiation (Dietrich & Krieger, 2009).

The schematic diagram and testicular structure in this fish was classified into two compartments, including germinal compartment (or seminiferous tubule) and interstitial compartments (Figure 4.6A-C). The interstitial compartment protruded from the tunica albuginea. Several compositions including Leydig cells, myeloid cell, fibroblast and blood vessels were observed (Figure 4.6B-C). Similar to previous researches Leydig cells had irregular shape and were present as a single or cluster cell, as previously agreed in many researchers (Billard, 1992; Dietrich & Krieger, 2009; Nagahama, 1983). The Leydig cell is considered to be the androgen producing cell in male fish, as confirmed in *Synbranchus senegalensis*, *Padogobius martensi* (Cinquetti & Dramis, 2003) and *S. marmotatus* (Nostro *et al.*, 2004). The germinal compartment consisted of regular branching seminiferous tubules and Sertolic cells. The sertoli cells usually extended their thin cytoplasmic processes and packed a germinal cyst-liked structure (Figure 4.6B). Within their cysts, several stages of sperm cells were present, as similar to previous studies (Billard, 1992; Dietrich & Krieger, 2009; Nagahama, 1983).

The testicular type was defined as restricted-spermatogonial type (Figures 4.6A-B). This type showed the distribution of spermatogonia to the distal end of the lobule. Similarly, it was generally observed in Phallostethid such as *Phenacostethus smithi*, *Phenacostethus posthon*, *Neostethus borneensis*, *Neostethus bicornis*, *Gulaphallus mirabilis* and *Gulaphallus bikolanus* (Grier & Parenti, 1994) and other atherinomorph fishes (Atheriniformes, Beloniformes and Cyprinodontiformes) (Grier *et al.*, 1980; Nagahama, 1983; Parenti & Grier, 2004; Uribe *et al.*, 2014). The spermatogenic process applied to the sequence of morphological and histological changes was clearly divided

into three distinct phases: spermatogonial, spermatocyte and spermiogenetic phases, as similarly to previous studies (Grier & Parenti, 1994; Mok & Munro, 1997).

The spermatogonial phase composed of different generations of spermatogonia that undergo mitotic divisions. Spermatogonia could be divided into two types: type A and type B spermatogonia (Figure 4.6A and 4.6D). Type A spermatogonia was locally seen in the distal end of the lobule of the testis. The large nucleus of Type A had a single nucleolus, which was surrounded by light granular cytoplasm. It is believed this type acted as the stem cell. Type B spermatogonia were smaller rather than type A spermatogonia. Type B divided more rapidly than the type A spermatogonia. The number of spermatogonia generations was about 3-14 generations in each cyst before entering meiosis, as suggested by (Schulz *et al.*, 2010). They replicated their chromosomes under meiosis before becoming spermatocyte phase.

The spermatocyte phase segregated by meiotic divisions, which divided into two cell cycles (primary and secondary spermatocytes) (Figures 4.6C-D). It was generally seen in male vertebrates (Uribe *et al.*, 2014). The characterization of the primary spermatocyte phase was ovoid shape and it has smaller cell when compared to spermatogonium. The gradual clumping of nuclear chromatins and the disappearance of nucleolus were prominently identified in this stage. Eosinophilic cytoplasm was restricted around their nucleus (Alberts *et al.*, 1994; Schulz *et al.*, 2010). The secondary spermatocyte was produced under mieosis I from the primary spermatocyte, therefore it was smaller than the primary spermatocytes (Schulz *et al.*, 2010). The nuclear characteristics of secondary spermatocytes was basophilic stain surrounding the scanty cytoplasm. The duration of this stage is short in most teleost (Billard, 1992; Nagahama, 1983).

The spermiogenetic phase had the morphological and functional changes, which related to the differentiation of spermatids to spermatozoa. The spermatid showed a high nuclear condensation (Figure 4.6C-D). Very scantly eosinophilic cytoplasm was also seen. It is agreed in some fish (Schulz *et al.*, 2010). Spermatozoa was the smallest cell size among the spermatogenic cells it is also called mature spermatozoa (Figures 4.6E-G). Under a light microscope it consisted of a head and tail (Figure 4.6E). Initially, both the head and tail of the spermatozoa were clumped together. After that, the

spermatozoa migrated to efferent duct and were released from the germinal cyst to the efferent duct system.

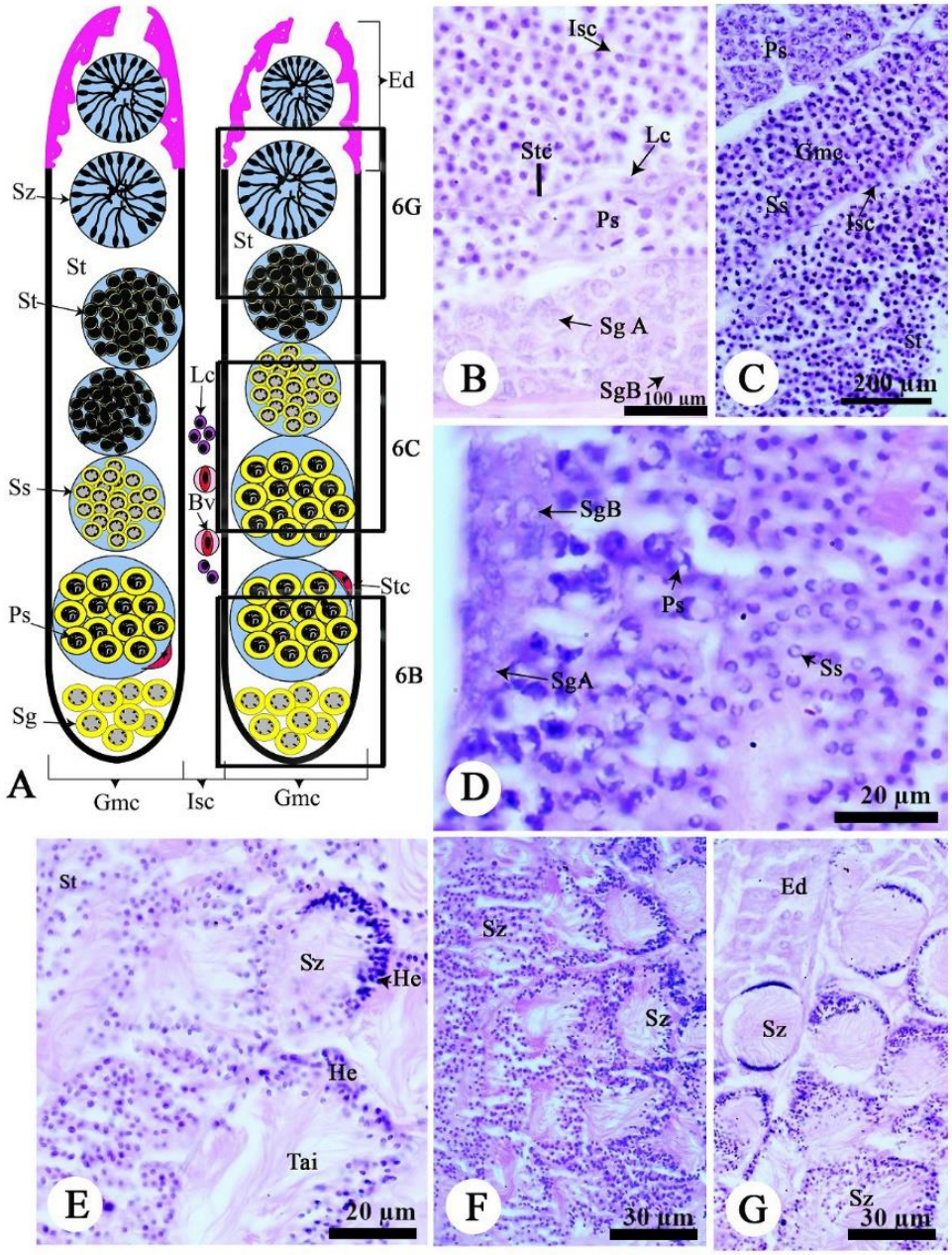

Figure 4.6 Schematic diagram and light microscope of the seminiferous tubules containing the different stage of sperm in *Neostethus lankesteri* collected from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center. **Abbreviations:** Bv = blood vessel, Gmc = germinal compartment, He = head of spermatozoa, Isc = interstitial compartment, Lc = Leydig cell, Ps = primary spermatocytes, Ss = secondary spermatocytes, SgA = spermagonium type A, SgB = spermagonium type B, St = spermatid, Stc = Sertoli cell, Stu = seminiferous tubule, Sz = spermatozoa, Tai = tail of spermatozoa.

Priapium organ

The priapium organ is the secondary sex character. It is an intromittent organ on the isthmus of adult males. The priapium organ of the *N. lankesteri* appeared as shiny white to transparent in live mature males. It was observed as a bone with positive to alizarin red staining (Figure 4.7B). The priapium organ merged from the pelvic fin and curved forward almost to the entire length of the head as similar to another Phallostethid such *N. bicornis* (Parenti, 2014). It is believed that the function of the priapium organ was for clasping and transferring bundle of sperm to the female during copulating (Mok & Munro, 1997; Parenti, 2014).

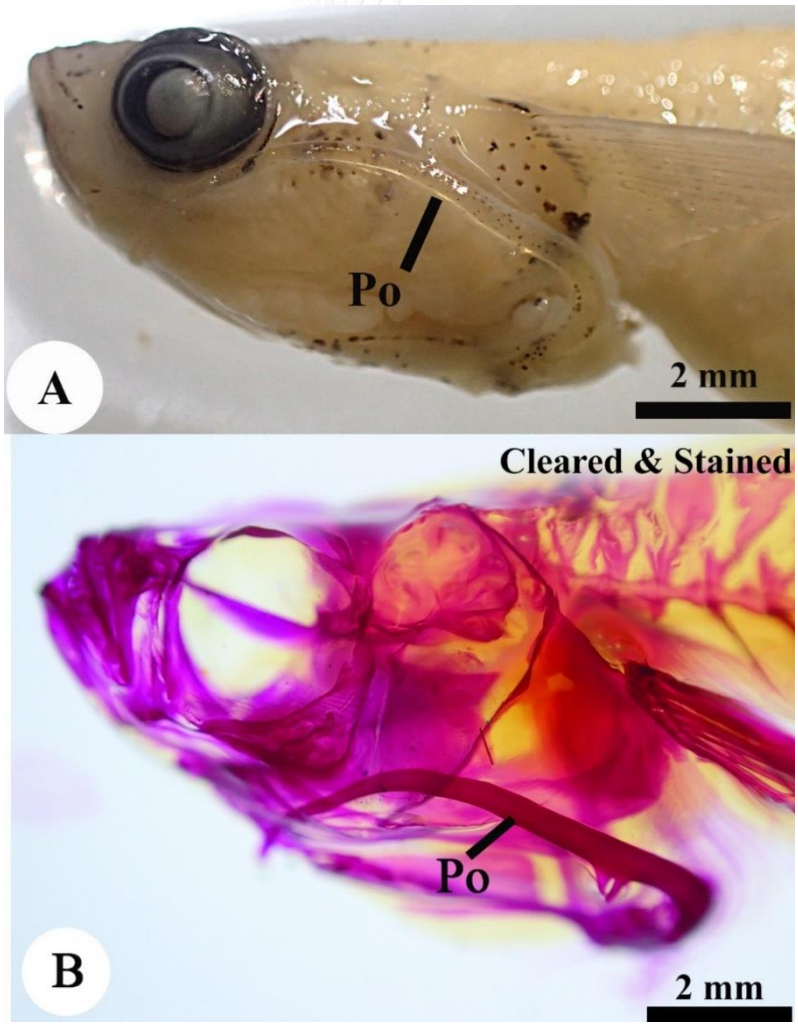

Figure 4.7 Morphology and cleared and stained of the priapium organ (Po) in *Neostethus lankesteri* from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center.

Ovarian differentiation of *Neostethus lankesteri*

In *Neostethus lankesteri* with standard length of 1.5 cm, ovary was found in the abdominal cavity. The primary growth phase (perinucleolar stage) was arranged in the ovarian structure. The ovary of *N. lankesteri* with standard length 1.6 – 1.7 cm was similar to previous stage. The primary growth phase including perinucleolar stage and cortical alveolar stage was found in the ovarian structure (Figure 4.8A-C). The ovary of *N. lankesteri* (1.8 – 1.9 cm SL) was similarly occupied as in previous stage. However, the beginning of secondary growth phase was found, two differential stages of oocytes including early secondary growth stage and late secondary growth stage were detected (Figure 4.8D-F). In 2.0 - 2.2 cm SL fish, the ovary of *N. lankesteri* had similar pattern as in previous stages with the presence of the primary growth and secondary growth phases. However, the additional and full-grown oocyte phase was also observed (Figure 4.8G-L). In fish with 2.3 - 2.4 cm SL, the ovary of *N. lankesteri* had the same pattern as in previous stages with the presence of the primary growth and secondary growth phases and the full-grown oocyte. However, atretic follicle oocytes were also observed (Figure 4.8M-O). Therefore, this study suggested that the fish at 1.8 cm SL was the size of first maturity.

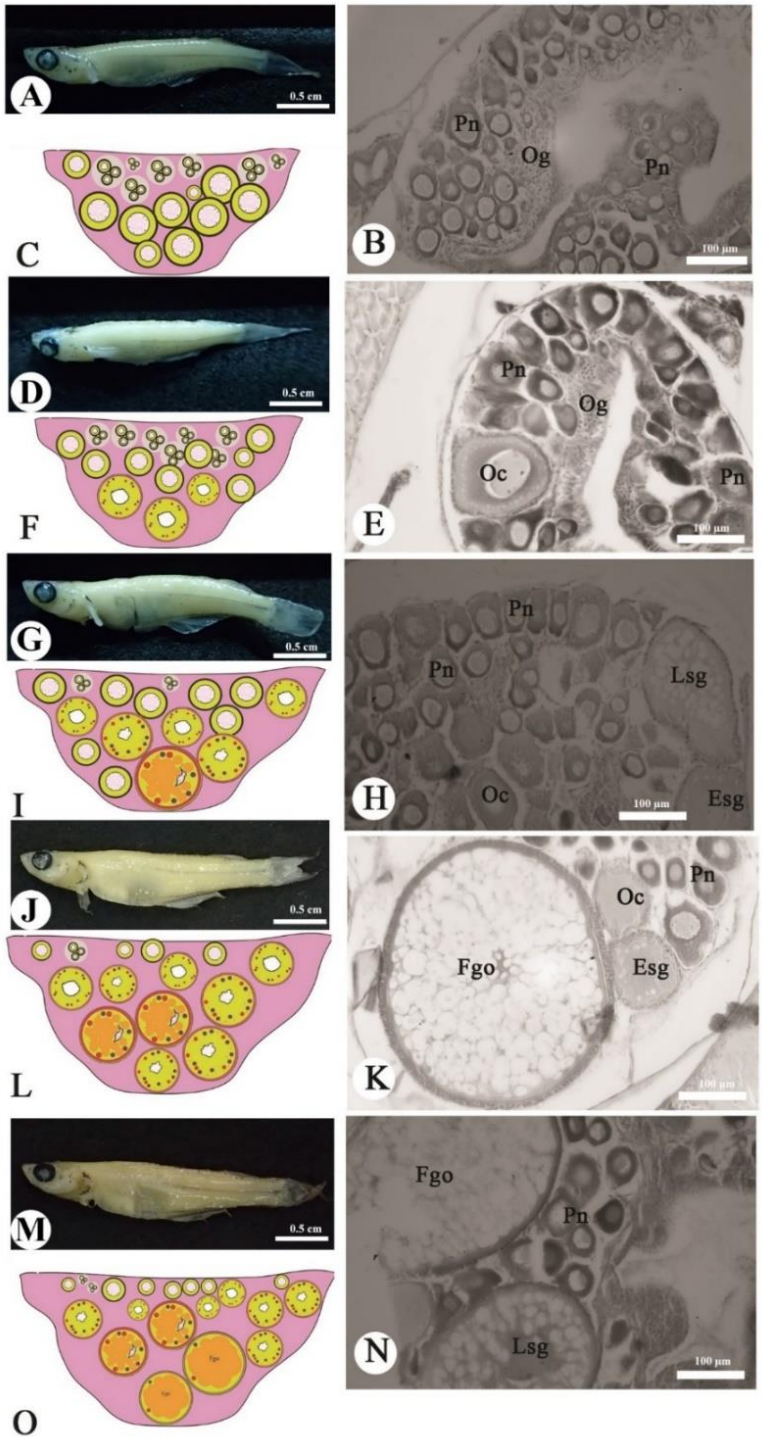

Figure 4.8 Morphology and light photomicrograph of the ovarian differentiation during 1.5 to 2.4 cm in standard length *in Neostethus lankesteri* from Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center. **Abbreviations:** Ego = early secondary growth step, Fgo = Full-grown oocyte step, Lsg = late secondary growth step, Oc = oil droplet and cortical alveolar stage, Og = oogonia and Pn = perinuceolar stage.

Conclusion

The present study revealed the gametogenesis of *Neostethus lankesteri* for the first time. In female *N. lankesteri*, the mature ovary in these fish was an asynchronous development oocyte type which consisting of two phases: primary growth phase (perinucleolar and oil droplet and cortical alveolar stages) and secondary growth phase (early vitellogenic, late vitellogenic and mature stages). Moreover, post-ovulatory phase was also observed in sampling periods. In male *N. lankesteri*, the mature testicular parenchyma was a restricted spermatogonial type. The spermatogenetic stage could be divided into three phases including spermatogonial, spermatocyte and spermiogenetic phases. The observation of post-ovulatory phase indicates the *N. lankesteri* was spending their entire life in the Pranburi River estuary or true resident species.

CHAPTER 5

GENERAL DISCUSSIONS AND CONCLUSIONS

The priapiumfish, *Neostethus lankesteri* is hypothesized as a link between primary producer and higher consumer in the areas of Pranburi River Estuary and Sirinaj Rajini Mangrove Ecosystem Learning Center (SRMELC), Prachuap Khiri Khan Province. Previous studied reports that *N. lankesteri* is the dominant species in both habitats. However, the roles and utilization of *N. lankesteri* in the ecosystem have not been any reported. Therefore, the understanding of the feeding ecology and reproductive biology in the aspect of gametogenesis.

The observation on the gut analysis of *N. lankesteri*, food items were categorized into four groups: diatoms, algae, dinoflagellates, and zooplankton. The index of relative importance, IRI showed that diatoms were a major component followed by zooplankton in Pranburi River estuary, whereas zooplankton was major component followed by diatoms in SRMELC during the two seasons. The nature of the spatial distribution of the food sources which is normally not uniformly distributed may influence the differences of the composition of food in gut content. Based on the gut content of *N. lankesteri* and the amount of zooplankton and phytoplankton found in its habitat, this study suggested that the feeding behavior of *N. lankesteri* is a generalist (*sensu stricto*) on zooplankton.

In addition to the stations at the estuary, we also sampled the station at the freshwater area at the Pranburi River. However, we did not get any *N. lankesteri* specimen from the station that was in freshwater condition. (Originally there were seven stations in this survey. However, none of the sample were collected from this site so we did not include it in the method). The digestive tract morphology of *N. lankesteri* has a superior mouth with canine teeth on premaxilla and small teeth on dentary. The pharyngeal toothplates was numerous and canine- shaped which functioned as a grinder for soft-preys species. Moreover, the low value of intestine coefficient (0.55) was considered as carnivorous intestine type. The results of the morphological study of digestive tract associated with the gut content analysis demonstrated that the *N.*

lankesteri was a carnivorous fish feeding on zooplankton. Others food component were considered as accidentally prey. The results of the histology of digestive system in *N. lankesteri* showed stomachless, which intestine tract could be divided into three regions: anterior intestine, middle intestine, and posterior intestine. However, overall histologically structures were generally not different from stomachless fish.

The histological investigation of gametogenesis of *N. lankesteri* revealed that in the female, the mature ovary in these fish was an asynchronous development oocyte type which consisting of two phases: primary growth phase (perinucleolar and oil droplet and cortical alveolar stages) and secondary growth phase (early vitellogenic, late vitellogenic and mature stages). Moreover, the post-ovulatory phase was also observed in sampling periods. In male *N. lankesteri*, the mature testicular parenchyma was a restricted spermatogonial type. The spermatogenetic stage could be divided into three phases including spermatogonial, spermatocyte and spermiogenetic phases. Based on gametogenesis of this species and the fact that *N. lankesteri* were not collected from freshwater station, *N. lankesteri* was a true resident species completing their life cycle in the Pranburi River estuary and Sirinaj Rajini Mangrove Ecosystem Learning Center.

These observations provided crucial information about habitat utilization of *N. lankesteri* in the Pranburi estuary ecosystem. *Neostethus lankesteri* utilized the ecosystem for both feeding ground and spawning ground. The *Neostethus lankesteri* shows a role in linking between primary consumer and higher consumer and may act as the intermediate of energy transfer from the lower trophic level to higher trophic level in the Pranburi estuary ecosystem.

Recommendations

Neostethus lankesteri has an asynchronous development oocyte type which suggested that they spawn year round. However, several asynchronous oocyte type fishes tended to have peak spawning period as well. Therefore, further study should examine the reproductive cycle of *N. lankesteri* in Pranburi River estuary and adjacent areas in order to understand the spawning periods of this species.

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APPENDIX

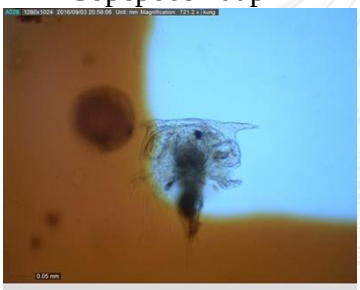
Appendix I. Some food items in the gut of *Neostethus lankesteri*

Hapacticoid copepod

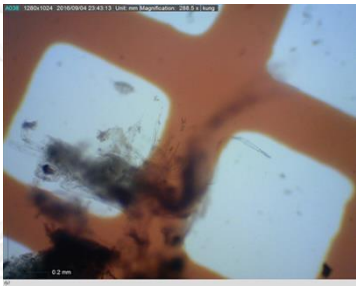
Ostracod

Copepods nuaplii

Part of insect



Cirripedia larva



Shrimp larva

Calanoid copepod

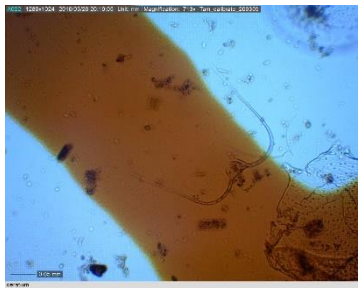
Centric diatom

Insect

Fish egg

Appendix I. Some food items in the gut of *Neostethus lankesteri* (Continue)

Noctiluca sp.



Ceratium

Tintinnid

Part of larvae crab

Zoea

Part of larvae shrimp

Nematode

Larvae insect

Part of larvae crab

Mollusk larvae

Appendix II. Sampling location of Pranburi River estuary (ST1-ST3) and Sirinaj Rajini Mangrove Ecosystem Learning Center (ST3-ST6)

Station 1

Station 2

Station 3

Appendix II. Sampling location of Pranburi River estuary (ST1-ST3) and Sirinaj Rajini Mangrove Ecosystem Learning Center (ST3-ST6) (Continue)

Station 4

Station 5

Station 6

Appendix III. Statistical analysis

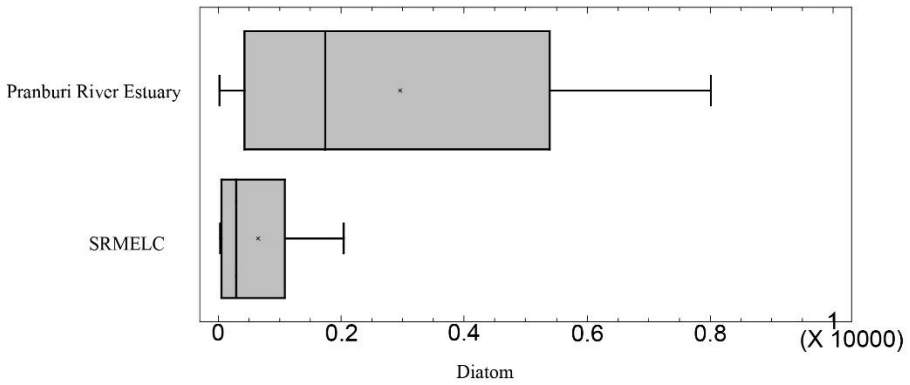


Figure 1. Availability of diatom in water columns between Pranburi River estuary and Sirinart Rajini Mangrove Ecosystem Learning Center during March 2015 to February 2016.

VITA

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International publications (In preparation)

1. Palasai, A., Na Lampang, P., Senarat, S., and Kettratad, J. Gut content and histo-histochemistry of the digestive system in Neostethus lankesteri Regan, 1916 and relationship with feeding type.

2. Palasai, A., Na lampang, P., Senarat, S., Jiraungkoorskul, W., and Kettratad, J. Reproductive pattern and ovarian differentiation of Neostethus lankesteri Regan, 1916 from Pranburi River estuary, Thailand.

National proceeding

Amphornphan Palasai, Phakorn Na Lampang, and Jes Kettratad. 2016. Preliminary study of diet composition in the priaprium fish Neostethus lankesteri Regan, 1916 from estuary of Pranburi River, Thailand. Proceeding of the 3rd National Meeting on Biodiversity Management in Thailand, 15-17 June 2016, Nan Province, Thailand. pp246 - 249.