HISTOLOGY OF TESTIS AND BROOD POUCH OF BROODING AND NON-BROODING MALE SEAHORSES, *HIPPOCAMPUS KUDA*

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ABSTRACT

This research describes the histology of the testis and the brood pouch of brooding and non-brooding male seahorses, *Hippocampus kuda*, during the reproductive season. Various spermatogenic cells: spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids were observed in both brooding and non-brooding males. Spermatogonia and primary spermatocytes were found along the entire length of the testis, while secondary spermatocytes and spermatids were found in the lumen, though no sperm were observed. Brooding and non-brooding males showed similar percentage of spermatogenic cells. Secondary spermatocytes were at the highest proportion, followed by primary spermatocytes, spermatogonia and spermatids, respectively. The brood pouches of brooding and non-brooding males were composed of outer (stratified cuboidal) and inner (pseudostratified) epithelia. Between them, there were 3 tissue layers: outer dense irregular connective tissue, middle smooth muscles and inner loose connective tissue layers. The inner loose connective tissue layer was thin and highly vascularized with large blood vessels in the brooding male while it was thick and vascularized with capillaries in the non-brooding male. The brood pouch consisted of 5 different stages: normal stage in non-brooding male (inner epithelium and tissue layer were thick and contained many small blood vessels), embryo carrying stage in brooding male (the inner epithelium seemed to be stretched), embryo released stage in brooding male (inner epithelium and tissue layer were flat and the surface showed rupture appearance), repair stage I in non-brooding male (inner epithelium and tissue layer were highly digitated) and repair stage II in non-brooding male (inner epithelial cells had become increased in size and inner tissue layer was filled with loose connective tissue).

KEY WORDS: TESTIS/ BROOD POUCH/ SEAHORSE/
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จุลกายวิภาคของอัณฑะและถุงเพาะฟักเอมบริโอในม้าน้ำเพศผู้ระยะเพาะฟักและไม่เพาะฟักเอมบริโอ, *Hippocampus kuda* (HISTOLOGY OF TESTIS AND BROOD POUCH OF BROODING AND NON-BROODING MALE SEAHORSES, *HIPPOCAMPUS KUDA*)

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บทคัดย่อ

งานวิจัยชิ้นนี้ได้กล่าวถึงจุลกายวิภาคของอัณฑะและถุงเพาะฟักเอมบริโอในม้าน้ำเพศผู้ *Hippocampus kuda* ระยะเพาะฟักและไม่เพาะฟักเอมบริโอ ในฤดูผสมพันธุ์ พบเซลล์สืบพันธุ์หลากหลายระยะ เช่น สเปอร์มาโทโกเนีย, ไพรมารี สเปอร์มาโทไซต์, เซกันดารี สเปอร์มาโทไซต์ และ สเปอร์มาทิดทั้งในอัณฑะของม้าน้ำเพศผู้ระยะเพาะฟักและไม่เพาะฟักเอมบริโอ สเปอร์มาโทโกเนียและไพรมารีสเปอร์มาโทไซต์พบได้ตลอดความยาวของอัณฑะ ขณะที่เซกันดารี สเปอร์มาโทไซต์ และสเปอร์มาทิดพบบริเวณกลางท่อของอัณฑะ แต่ไม่พบอสุจิเลย เปอร์เซ็นต์เซลล์สืบพันธุ์แต่ละระยะในม้าน้ำเพศผู้ระยะเพาะฟักและไม่เพาะฟักเอมบริโอมีความคล้ายคลึงกัน คือมีเซกันดารี สเปอร์มาโทไซต์ เปอร์เซ็นต์สูงสุด รองลงมาคือ ไพรมารี สเปอร์มาโทไซต์ ตามด้วยสเปอร์มาโทโกเนีย และสุดท้ายคือ สเปอร์มาทิด ถุงเพาะฟักของม้าน้ำเพศผู้ระยะเพาะฟักและไม่เพาะฟักเอมบริโอ ประกอบด้วยชั้นเนื้อเยื่อบุผิว 2 ชั้น คือ เนื้อเยื่อบุผิวชั้นนอก (ชั้นเซลล์ลูกบาศก์ซ้อนกัน) และ เนื้อเยื่อบุผิวชั้นใน (ชั้นเซลล์ซ้อนกันแบบเทียม) ในระหว่างชั้นเนื้อเยื่อบุผิวทั้งสองประกอบด้วยเนื้อเยื่อ 3 ชั้น คือ เนื้อเยื่อชั้นนอก (เนื้อเยื่อเกี่ยวพันแบบแน่นทึบ), เนื้อเยื่อชั้นกลาง (กล้ามเนื้อเรียบ) และเนื้อเยื่อชั้นใน (เนื้อเยื่อเกี่ยวพันแบบโปร่ง) เนื้อเยื่อชั้นในของถุงเพาะฟักในม้าน้ำระยะเพาะฟักเอมบริโอมีลักษณะบาง ประกอบไปด้วยเส้นเลือดจำนวนมาก ในขณะที่ ถุงเพาะฟักของม้าน้ำระยะไม่เพาะฟักเอมบริโอมีลักษณะหนาประกอบด้วยเส้นเลือดฝอยจำนวนน้อย ถุงเพาะฟักของม้าน้ำมี 5 ระยะ คือ ระยะปกติ (เนื้อเยื่อบุผิวและเนื้อเยื่อชั้นในมีลักษณะหนาประกอบด้วยเส้นเลือดขนาดเล็ก), ระยะมีเอมบริโอยู่ (เนื้อเยื่อบุผิวมีลักษณะเสมือนยืดออก) ระยะที่เอมบริโอหลุดออกไปแล้ว (เนื้อเยื่อบุผิวและเนื้อเยื่อชั้นในมีลักษณะบางเป็นริ้วย่น), ระยะซ่อมแซมที่ 1 (เนื้อเยื่อบุผิวและเนื้อเยื่อชั้นในมีลักษณะคล้ายนิ้วมือ), ระยะซ่อมแซมที่ 2 (เนื้อเยื่อบุผิวและเนื้อเยื่อชั้นในมีขนาดหนาขึ้น)

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LIST OF ABBREVIATIONS

AnSc	Anaphase spermatocyte
BV	Blood vessel
CB	Coiled blood
°C	Degree Celsius
cm	centimeter
E_2	Estradiol
EM	Embryo
et al.	And other
g	gram
h	hour
IEP	Inner epithelium
IL	Inner tissue layer
LBV	Large blood vessel
LSc	Leptotene spermatocyte
MSc	Metaphase spermatocyte
µm	micrometer
ML	Middle layer
ml	milliliter
mm	millimeter
OEP	Outer epithelium
OL	Outer tissue layer
PSc	Pachytene spermatocyte
%	Percent
P	Progesterone
Psc	Primary spermatocyte
SBV	Small blood vessel
SE	Standard error
Ssc	Secondary spermatocyte

LIST OF ABBREVIATIONS (CONT.)

Sg	Spermatogonia
St	Spermatid
T	Testosterone
TGC	Triangular giant structure
Y	Yolk
ZSc	Zygotene spermatocyte

CHAPTER 1

INTRODUCTION

Seahorses are bony fishes (teleosts) and are classified in the same family as pipefishes (genera *Nerophis* and *Syngnathus*), sea dragons (genera *Phycodurus* and *Phyllopteryx*) and pipehorses (genera *Solegnathus* and *Acentronura*) (Vincent et al., 1992). A total of 32 species of seahorses is established worldwide. There are 5 species of seahorses in Thailand, *Hippocampus kuda, H. trimaculatus, H. spinosissimus, H. mohnikei* and *H. histrix*. Approximately 20 million seahorses are collected from the wild annually (Vincent and Hall, 1996). The total global consumption of seahorses was at least 20 million (more than 56 metric tones) in 1995 and 25 million (more than 70 metric tones) in 2001. Many seahorse species are included as "vulnerable" on the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Animals (Louire et al., 1999). Seahorses are threatened by heavy exploitation for use as traditional medicines, aquarium fishes and souvenirs. More than 95% of the captured seahorses are used in traditional Chinese medicine. Seahorses and their relatives are used to treat a variety of illnesses, from asthma, arteriosclerosis to incontinence and impotence. They also provide remedies for skin ailments, high cholesterol levels, excess throat phlegm, goiters, heart disease and lymph node disorders (Vincent and Hall, 1996). Most dead seahorses are probably imported to China, Hong Kong and Taiwan. In addition, seahorses are used in traditional Jamu medicine in Indonesia and Folk medicine in the Philippines. In the United States, Australia and the surrounding nations, seahorses are used for tourist industries. They are used to make souvenirs such as key chains, to incorporate into jewelry and other crafts. In the United States, both live and dried seahorses are imported mostly from the Philippines for aquarium use. In 1987, about 200,000 seahorses were imported from the Philippines. In Australia, a live aquarium fish may be sold for $20 (Vincent, 1995). Another threat to seahorses is the degradation and destruction of their habitats, i.e., coral reef, mangrove, seagrass,

through human activities. Pollution and development of marine industries have also contributed to the pressure on the survival of seahorses. A 15-50% seahorses decline in the past 5 years and a 70% decline over the past 10 years were recorded in the largest seahorse exporters, e.g., Thailand, Vietnam, India and the Philippines. The consequence of seahorse exploitation is that seahorses become an endangered species (Louire et al., 1999).

Researchers have focused on study of ecology of seahorses and on rearing to partial replenishment of depleted wild stock. Studies on behavior, feeding and biology of the lined seahorse, *H. erectus* (Teixeira and Musick, 2001) and on monogamous pair bonds and mate switching in the Western Australian seahorse, *H. subelongatus* (Kuarnemo, 2000) have been conducted. Field observation on tagged seahorse, *H. comes* in the Philippines discovered that adult seahorses were nocturnal, maintained small home ranges and lived mostly among corals (Perante, 2002). Morphology, anatomy and prey capture in the lined seahorse have been studied (Bergert and Wainwright, 1997). Taxonomy of the Western Indian Ocean seahorse, *H. fuscus* in the Mediterranean was recorded (Golani and Fine, 2002) and a new species of pygmy seahorse, *H. denise* from Indonesia was found (Lourie and Randall, 2003). Hatchery and rearing of juveniles in, *H. abdominalis* (Woods, 2000) and culture of the spotted seahorse, *H. kuda* (Job et al., 2002) were reported but few research in reproduction has been carried out.

The spotted seahorses are the largest seahorses in Thailand. They are generally found in a broad range of shallow inshore habitats. They are highly valued seahorses that are popular in both traditional medicines and marine aquariums trades and are one of the most heavily traded seahorse species in the Southeast Asian countries (Lourie et al., 1999). Studies of *H. kuda* mostly involve hatchery rearing to improve survival, while the reproductive biology such as gonad development is poorly understood. Understanding the reproductive biology of seahorse is essential for seahorse cultivation and conservation. Carcupino et al. (1999) studied testis structure and spermatogenesis of pipefishes, *Syngnathus abaster* and *S. acus* during reproductive period. The hollow paired testes contained different spermatogenic cells: spermatogonia, primary spermatocytes, developing symplastic spermatids, large droplet-containing cells and spermatozoa. Secondary spermatocyte was not observed.

Ultrastructure of brood pouch of the pipefishes, *Nerophis ophidion* and *S. abaster* and the seahorse, *H. hippocampus* has been reported (Carcupino, 2002). In *H. hippocampus*, the brood pouch skin consisted of two layers: epidermis and dermis. The epidermis was swollen epidermal cells. The dermis composes of many superficial large capillaries. Watanabe et al. (1999) presented the characteristics of the brood pouch epithelial cells in the pipefish, *S. schelegeli*. The epithelial cells consisted of mitochondria-rich cells and pavement cells. The objectives of the present work were to study changes in the cellular structure of the testis and the brood pouch in brooding and non-brooding spotted male seahorses during reproductive season.

CHAPTER 2

OBJECTIVES

The objectives of the present study were as follows:

1. To study testicular development in brooding and non-brooding male, *H. kuda.*

2. To study morphological alteration of the brood pouch during brooding in the male, *H. kuda*.

CHAPTER 3

LITERATURE REVIEW

1. Biology of *H. kuda*

1.1 External Morphology

Seahorses are morphologically unusual fish. Their heads are bent at right angles to their body; the trunks are inherently curved; the bodies are armour-plated and the tails are prehensile (Lourie et al., 1999). The bodies of seahorses are slender and scale-less. The bony plates are arranged in series of rings. A small oblique mouth is located at the end of a tubular snout. Seahorses have no teeth and suck their preys (foods) in the tubular snouts (Muller and Ossae, 1984). Their eyes move independently allowing them to maximize the search areas. They also lack stomachs and thus must consume large quantities of food to compensate for their rapid and inefficient digestion (Lourie et al., 1999). Seahorses retain only a subset of fins found in most other fish. They have a dorsal fin and two tiny pectoral fins but lack pelvic and caudal fins, and have only a tiny anal fin. Only male seahorse has a pouch for embryo brooding.

1.2 Taxonomy

Spotted seahorses, *H. kuda,* occupy a taxonomic position within:

Phylum	Chordata
Class	Actinopterygii
Order	Syngnathiformes
Family	Syngnathidae
Subfamily	Hippocampinae
Genus	*Hippocampus*

They are the largest seahorses in Thailand and are highly commercialized for aquarium fish. Their color is often totally black with a graining texture. Sometimes, they may become pale yellow or creamy color with fairly large, dark spot (especially

in the females). The color may be sandy, blending in with the surrounding. An adult length varies from 7.0 to 17.0 cm. There are 34-36 rings around the trunk. The average snout length is 2.3 cm. The characteristic of coronet is a low-medium, rounded structure overhanging at the back, often with a cup-like depression on the top; sometimes, there are no spiny broad flanges. The spines are low, rounded bumps. Other distinctive characteristic is a thick snout (Figure 1). They are distributed throughout the Indo-Pacific (from Pakistan and India to Southern Japan and Hawaii) (Table 1). Their habitats are seagrass and marine algae areas of estuaries and seaward reef. They are found in open water and attached to drifting *Sargassum* up to 20 km from shore. Their environment is a normal brackish marine (Lourie et al., 1999).

1.3 Habitats and Distribution

All seahorses are marine species, generally live among seagrass beds, mangrove roots, coral reefs and estuaries. Most seahorses are found near shore. Some species can tolerate a wide range of salinity. Seahorses are distributed globally in both tropical and temperate marine water where temperature ranges roughly from 45^{o} North to 45^{o} South latitudes. Most species are distributed in the West Atlantic and the Indo-Pacific regions. The most populated areas for seahorses are Southern Australia, Tasmania, China and the Philippines (McAllister, 1990). Seven species of seahorses found in the coastal water of Vietnam are *H. spinosissimus, H. comes, H. trimaculatus, H. kuda, H. kelloggi, H. mohnnikei* and *H. histrix* (Lourie et al., 1999). *H. abdominalis* is found in New Zealand (Woods, 2000). *H. zosterae* is found in Florida, USA (Masonjones, 2001). *H. comes* is found in the central Philippines (Perante, 2002) and *H. fuscus* is found in the eastern Mediterranean (Golani and Fine, 2002).

1.4 Food and Feeding

Seahorses are voracious predators, relying entirely on live moving food. They eat anything small enough to fit into the mouth. The main food source of seahorse is small crustaceans. In the wild, *H. ingens* feed on zooplankton, mainly tiny larvae of invertebrates and fish that bathe their habitats from upwelling ocean current (Job et al., 2002). *H. hippocampus* feed on small preys (zoobenthos, benthic invertebrate, crustacean and mysis) and organ debris (detritus). *H. abdominalis* feed on crustacean

Figure 1. Male and female of *H. kuda* (from Louire et al., 1999).

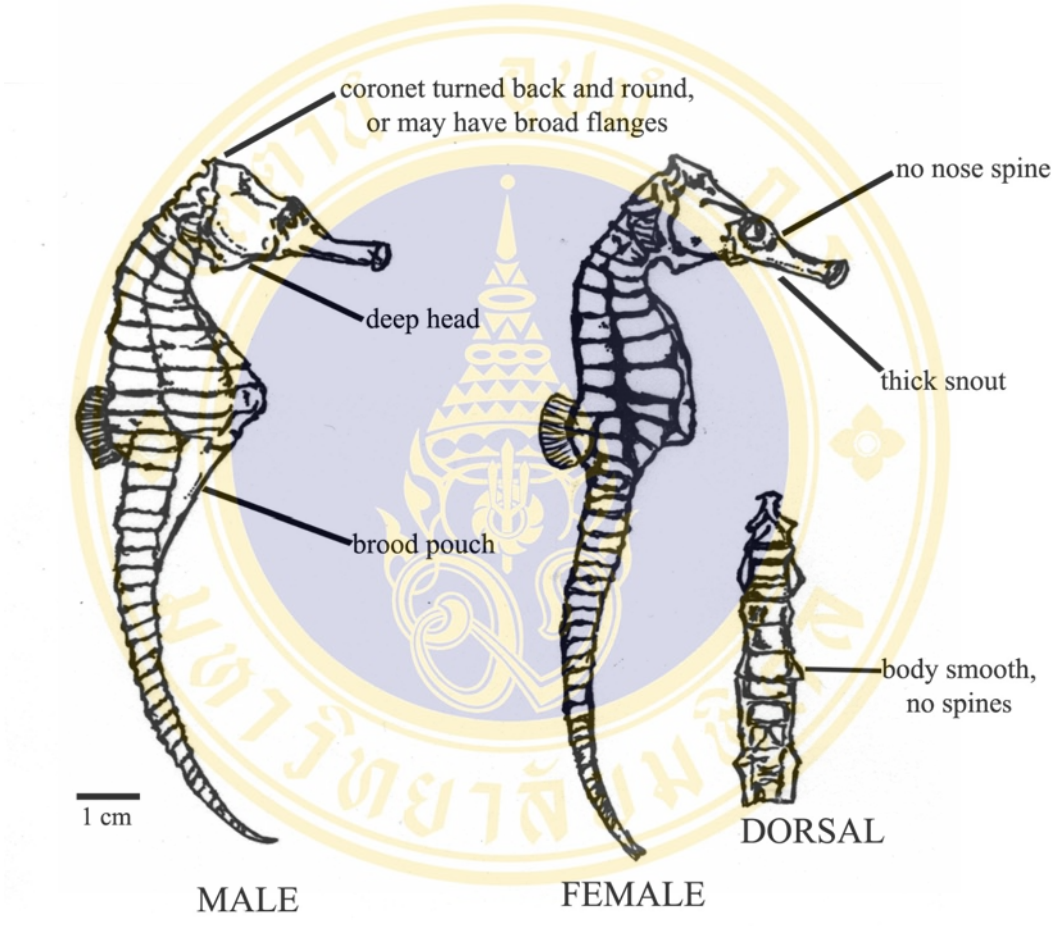


Table 1. Occurrences of *H. kuda* (from Lourie et al., 1999).

Country	Locality	Year	Author
Australia	New South Wales	1974	Dawson C.E.
Australia	New South Wales	1981	Dawson C.E.
Australia	New South Wales	1982	Dawson C.E.
Egypt		1933	Fourmanoir Et Postel
Hawaii	Honolulu	1932	
Hawaii	Kaula Island	1951	Heraid E.S.
Hawaii	South of Kauai	1960	Frizsche R.A.
India	Ennur	1941	Herre A.W.
Indonesia	Tobelo	1981	Dawson C.E.
Malaysia	Malaya	1961	
Malaysia	Malaya	1962	
Malaysia	Pulau Pisang-island	1937	
Malaysia	Malaysia	1953	
Malaysia	Malaysia	1953	
Malaysia	Siglap	1940	Herre A.W.
Mozambique	Booill	1964	
Mozambique	Delagoa Bay	1920	
Mozambique	Pinda Inland	1956	
Papua	Gorror Reef	1933	
Papua New Guinea	Kimbe bay New Britain	1996	Alen G.R.
Papua New Guinea	Nagada Harbour	1987	Crochet N.
Philippines	Cebu	1931	
Philippines	Dumaguete	1931	
Philippines	Grounds	1946	
Philippines	Mactan Island	1981	
Philippines	Corregidor	1947	Heraid E.S.
Saudi Arabia	Arabian Gulf	1982	Dawson C.E.
Singapore	Singapore	1952	
Singapore	Singapore	1955	
Singapore	Singapore	1947	Herald E.S.
Singapore	Singapore	1840	
Solomon Island	Purvis Bay	1945	Harry R.R.
South Africa	Durban	1919	
South Africa	Durban	1920	
South Africa	Durban	1936	
South Africa	Keurbooms estuary	1973	
USA	La Perouse bay	1972	Dawson C.E.
USA	Kaneoke bay	1972	
Vanuatu	New Hebrides	1926	
Vanuatu	New Hebrides	1926	
Vanuatu	New Hebrides	1931	
Viet Nam		1863	
Viet Nam	Nam Phan (Cochin China)	1949	

crustaceans, such as copepods and amphipods (Louire et al., 1999).

1.5 Predator

Young seahorses are vulnerable to predators, most notably piscivorous fishes. Adult seahorses do not have many natural predators, probably because they are difficult to find and relatively unpalatable owing to their bony plates and spiny structures. Seahorses have been found in the stomachs of pelagic fishes e.g. tuna, red snapper, eldorado (dolphin fish), crabs and seabirds (even penguins). For some seahorses, humans are the greatest predators (Louire et al., 1999).

1.6 Reproduction

A very unique feature of the family Syngnathidae is the presence of a brood pouch in the male. Male sexual maturity is signified by the presence of a brood pouch on the tail below the abdomen. Females appear to mature at much the same size as males. On the average, seahorses reach sexual maturity between 6 months and 1 year of age (Vincent and Hall, 1996). Pair of seahorses usually go through courtship rituals for several days before mating. After courtship, the female inserts her ovipositor into the male pouch and deposit eggs. The eggs are fertilized by the male and are embedded in the pouch wall (Herald, 1959). The pouch acts like a womb, providing nutrients and oxygen to the embryos and removing wastes. The pouch seals shut after mating and the male broods the developing embryos. Oxygen diffuses from capillaries in the pouch tissue that envelops the eggs. Hormones help to create a placental fluid that bathes a small part of the egg that protrudes from the enveloping tissues into the pouch center. The pouch environment is altered during pregnancy from being similar to body fluid to resembling the surrounding seawater, presumably to reduce stress to the young at birth (Vincent, 1995).

The pregnancy lasts 10 days to 6 weeks, depending on the species and water temperature. At the end of gestation, the male goes into labor (at night) pumping and thrusting for hours to release his broods. The young are miniature replicas of their parents. They are fully independent and are able to feed for themselves immediately upon birth. Newborns of most species measure between 7 and 12 mm. Most species produce 100-200 youngs per pregnancy, although smaller species *H. zosterae* may

release only about five offsprings. The maximum known brood size for seahorses is 1,572 youngs (Vincent, 1990).

2. Testicular Development

2.1 Testicular structure

The male reproductive system consists of testis, accessory glands and sperm ducts. Most of the teleost fishes have similar parts of reproductive system. In South American catfishes, *Trachelyopterus lucenai* and *T. geleatus*, the male reproductive system can be classified into 4 main regions which are spermatogenic region, sperm storage region, secretory and storage regions of the seminal vesicles (Meisner et al., 2000). Chinabut et al. (1991) suggested that the male reproductive system of walking catfish, *Clarias batrachus,* is composed of testis, ductus efferens and genital orifice. However, the testicular morphology of the teleosts shows variation which may be short, tubular or lobular with their apices at the center of the organ (Weisei, 1949). The testicular feature of *T. lucenai and T. galeatus* is numerous finger-like lobes, while the testes of Atlantic halibut, *Hippoglossus hippoglossus*, are organized into branching lobes (Weltzien et al., 2002). However, the testis shapes of *C. batrachus, S. abaster, S. acus* and *Trachelyopterus bifasciatum* are elongated paired lobes (Chinabut et al., 1991; Carcupino et al., 1999; Koulish et al., 2002).

The testis lobule of *C. batrachus* is covered with a thin tunica albuginea, a dense white connective tissue layer, and with a distinct smooth muscle bundle layer. The testis composes of blood vessels and branching seminiferous tubules (Chinabut et al., 1999). In the pipefishes, *S. abaster* and *S. acus*, the testis lobule is encapsulated by the external vascularized tunica albuginea enveloping a tripartite germinal epithelium and has a single seminiferous tubule. Different spermatogenic cells are supported by the basement membrane (Carcupino et al., 1999).

The teleost testes can be divided into 2 types based upon the distribution of spermatogonia in the testes: restricted and unrestricted-spermatogonia testes (Grier et al., 1980). In the restricted spermatogonia-testes, the spermatogonia appear at the distal ends of the lobule while in the unrestricted-spermatogonia testes, the spermatogonia distribute along the lobule length (Selman and Wallace, 1986). The unrestricted-spermatogonia testes are typical type of most teleosts, e.g., Japanese

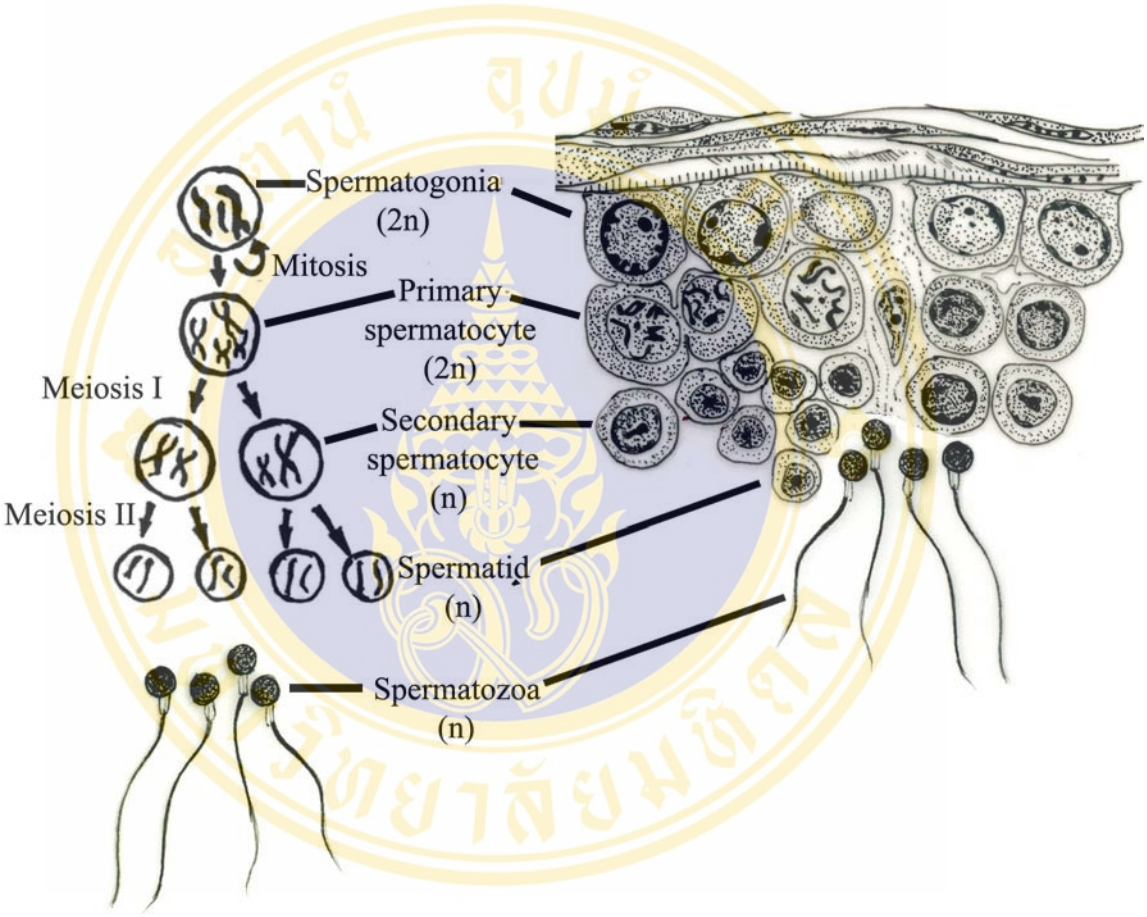
huchen, walking catfish and pipefish (Amer et al., 2001; Chinabut et al., 1991; Carcupino et al., 1999).

2.2 Testicular and spermatogenic cell development

The testes serve two main functions. They produce spermatozoa and androgen, especially testosterone. In the adult, secretion of testosterone is essential for the maintenance of sperm production (spermatogenesis) and for the maintenance of secondary sex characteristics, the genital duct, and the accessory glands. Spermatogenesis is the process by which spermatogonia divide and differentiate into spermatozoa. The process of spermatogenesis can be divided into three distinct phases (Rasquin and Halfter, 1951). Firstly the spermatogonial stem cells undergo mitotic proliferation leading to both new stem cells and differentiated spermatogonia. Secondly, the differentiated spermatogonia undergo meiosis, which eventually leads to haploid spermatids. Thirdly the spermatids differentiate into flagellated sperm. Most teleosts consist of 5 main stages of spermatogenic cells: spermatogonia, primary and secondary spermatocytes, spermatids and spermatozoa (Figure 2). In Salmonids, spermatogonial stem cells divide to produce generations of spermatogonia, which enter the spermatogenic cycle. Spermatogonia type A divide several times to produce spermatogonia type B which in turn develop into primary spermatocytes and then undergo meiosis to produce spermatids. The spermatids proceed through a morphological metamorphosis during spermiogenesis to spermatozoa (Billar, 1982). In contrast, 4 stages of the spermatogenic cells are observed in pipefishes: spermatogonia, primary spermatocytes, symplastic spermatids and spermatozoa; secondary spermatocyte was never observed (Carcupino et al., 1999).

From previous studies, the structures of spermatogenic cells in various teleosts are not completely different. The spermatogonia are large with various shapes, i.e., round in spotted halibut (Koya et al., 2003) or irregular shape in walking catfish (Chinabut et al., 1991). They contain large distinct nucleus with lightly stained granules and a prominent nucleolus. The 1-2 nucleoli can be observed in spermatogonia of the cichild fish (Fishelson, 2003). The cell membrane and cytoplasm are commonly indistinct and pale. The primary spermatocytes are large with varying sizes at their meiotic stages. The nucleus is spherical shaped. The

Figure 2. Fish seminiferous tubule showing spermatogenesis (from Chinabut et al., 1991).



cytoplasm contains darkly stained granules (Chinabut et al., 1991; Koya et al., 2003; Hayakawa et al., 2002). The secondary spermatocyte is smaller than the primary spermatocyte. Its nucleus is small and contains dark chromatin (Chinabut et al., 1991; Koya et al., 2003; Fishelson, 2003). The spermatids are smaller in size. They contain oval nucleus with more condensed chromatin (Koya et al., 2003; Koulish et al., 2002; Fishelson, 2003; Hayakawa et al., 2002). The chromatin appears to migrate to the periphery of the nuclear membrane in the walking catfish (Chinabut et al., 1991). In some cases, the distribution of granules is observed in nucleus (Fishelson, 2003). With ultrastructural study, the nuclear pores are observed in the nuclear membrane (Hayakawa et al., 2002). The spermatozoa consist of head and tail. The head containing chromosomal material is darkly stained and either round (Koya et al., 2003; Chinabut et al., 1991) or slightly elongate (Fishelson, 2003). The tail is composed of long flagellum that is indistinct via light microscope (Koya et al., 2003; Hayakawa et al., 2002).

2.3 Spermatogenic cycle

The sexual cycle can be defined into a part of life cycle in fish. The feeding of the fish during the warm season leads to growth of the body and deposition of fat in the mesenteries. Different teleosts show variation in spawning season and spermatogenic cell types in the testes (Table 2).

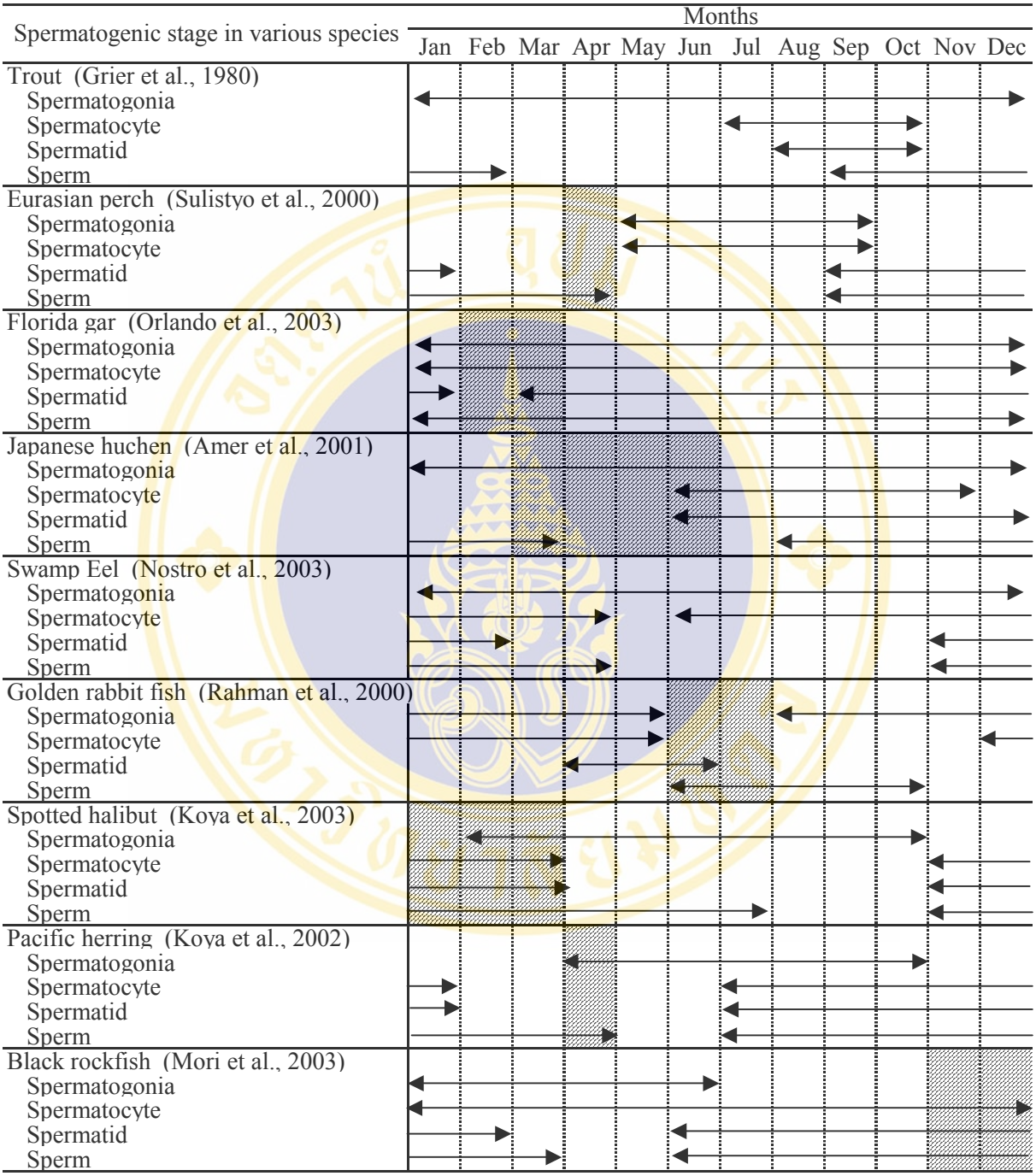
The percentage of spermatogenic cells is similar in some teleosts (Table 3). In general, the spermatozoa are highly abundant in testis lumen and sperm duct during spawning season while other spermatogenic cells (spermatogonia and spermatocyte) are present in small number. However, Florida gar, *Lepisosteus platyrhincus*, and Eurasian perch, *Perca fluviatilis*, have relatively equal proportion of spermatogenic cells in the testes (Orlando et al., 2003; Sulistyo et al., 2000).

3. Sex Hormones and Reproduction

3.1 Sex hormone structures and functions

Reproduction in vertebrates is usually seasonal or cyclical. Timing is crucial because the youngs should appear when food is available and environmental conditions are optimal for survival. Sexual activity is intrigued once set in motion by

Table 2. Reproductive period and spawning season in different teleosts.



arrow represents reproductive period.
shaded area represents spawning season.

Table 3. Percentage of spermatogenic cells in the testes of some teleosts during spawning season.

Fish species	Frequency of spermatogenic cells				References
	SG	SC	ST	SZ	
Japanese huchen (*Hucho perryi*)	+	-	-	+++	Amer et al., 2001
Golden rabitfish (*Siganus guttatus*)	-	0.3%	2.7%	97%	Rahman et al., 2000
Blackrock fish (*Sebastes schlegeli*)	+	-	-	+++	Mori et al., 2003
Pacific herring (*Clupea pallasii*)	-	-	-	>95%	Koya et al., 2002
Spotted halibut (*Verasper variegatus*)	-	<20%	-	>70%	Koya et al., 2003
Atlantic halibut (*Hippoglossus hippoglossus*)	-	-	-	+	Weltzien et al., 2002
Florida gar (*Lepisosteus platyrhincus*)	+	+++	++	++	Orlando et al., 2003
Eurasian perch (*Perca fluviatilis*)	++	++	++	++	Sulistyo et al., 2000

SG = Spermatogonia, SC = Spermatocyte,
ST = Spermatid, SZ = Spermatozoa,
- = No reported data, + = Low frequency,
++ = Medium frequency, +++ = High frequency.

some environmental cues, such as seasonal change in temperature, photoperiod, or some social force. Sexual reproductive process is also controlled by hormones. Estrogens are sex steroid hormones, controlled by the pituitary gonadotopins, follicle-stimulating hormone (FSH) and luternizing hormone (LH). The gonadotopins are in turn governed by the releasing hormone produced by neurosecretory center in the hypothalamus. The important sources of estrogen are ovary, adrenal cortex, Leydig cells of the testis. The relative potency of estrogens varies somewhat depending upon the method of bioassay used. Estradiol-17β is considerably the most potent form; estrone is the second in potency and estriol is relatively weak (Gray and Bacharach, 1967).

Testosterones are steroid hormones secreted by interstitial cells in the testes; only a small portion of the circulating level is from the peripheral conversion of precursor steroid secreted by the adrenal cortex. Testosterone is necessary for growth and development of the male accessory sex structures, development of secondary male sex characteristics and male sexual behavior. The testicular development and the testosterone secretion are controlled by FSH and LH (Hickman et al., 1993). In female, testosterone level is normally found to be much lower than the male. In fish, testosterone is produced by the testes. 11-Ketotestosterone (11-KT) and testosterone take part in the control of spermatogenesis in goldfish (Gray and Bacharach, 1967). The 11-KT hormone has been associated with the development of gametes and secondary sex characteristics such as coloration and behavior (Liley, 1969). In Japanese sardine, *Sardinops melanostictus*, the 11-KT acts as primary androgen working in testicular development (Matsubara et al., 1992). In many fishes, the 11-KT regulates spermatogenesis, e.g., striped bass and Japanese huchen (Amer, et al., 2001; Koya et al., 2003; Holland et al., 2000; Weltzein et al., 2002; Miura et al., 2002).

Progesterones are steroid hormones. The ovary and placenta are the major production sites but small amount is synthesized by the adrenal cortex in both male and female (Gray and Bacharach, 1967). In teleosts, progesterone has many functions, e.g., promotes milt production in Pacific herring (Koya et al., 2003), controls final maturation stage and plays a role in mitotic phase and meiotic process, regulates spawning behavior in Japanese huchen (Amer et al., 2001), regulates sperm

maturation and spawning in golden rabbit fish (Rahman et al., 2000), participates in final oocyte maturation in female, induces the acquisition of sperm mortality and elevation of pH in seminal plasma in male black rockfish (Mori et al., 2003).

3.2 Sex hormonal level during spawning season

Teleosts show variation in spawning season (Table 2), and sex hormone levels (Table 4).

4. Brood Pouch

Embryos of teleosts are taken care by female, male or both. For Syngnathidae, the embryos are cared by only male called male parental care (Blumer, 1982). The form of the parental care of this family is using brood pouch located on the abdomen or tail of the male. Although brood pouch is observed only in the Syngnathidae family, it has variation in morphology and function for parental care. The differences of the brood pouches are location and characteristics of the pouch. This variation is used for classification of a taxonomic grouping within the family (Herald, 1959). As a primitive brood pouch, egg of the snake pipefish, *Entelurus*, and worm pipefish, *Nerophis* are loosely attached to the ventral side of their trunk and are completely unprotected by the brood pouch. In pipehorse, *Solegnatus* and ringed pipefish, *Doryrhamphus*, the eggs are placed into individual membranous egg compartments. At the beginning of the brood pouch evolution, the eggs of *Ootethus* are incubated in a well-defined pouch and protected by pouch plate. In *Syngnathus*, the eggs are placed into a well-defined pouch with fleshy bilateral pouch folds. At high evolution of the brood pouch, the eggs of *Hippocampus* are incubated in a completely enclosed sac-like fleshy pouch, which opens externally through an anterior pore (Herald, 1959). Carcupino (2002) studied ultrastructural organization of the brood pouches in *S. abaster* and *H. hippocampus*. In *S. abaster*, the brood pouch skins are bilayer epithelia. The outer epithelium is composed of two different cell types; the classic filament-containing cells and the mitochondria-rich cells. The dermis (beneath the epithelium) is vascularized with capillaries. In *H. hippocampus*, the brood pouch skins are thin and folded. The epidermal cells are large and composed of distinctive features, i.e., dilation of cisternae in rough endoplasmic reticulum and concentration of tonofilaments in peripheral cells. The dermis contains large capillaries.

Figure 3. Pathway of sex steroid hormones, Estradiol, testosterone and progesterone (Gray and Bacharach, 1967).

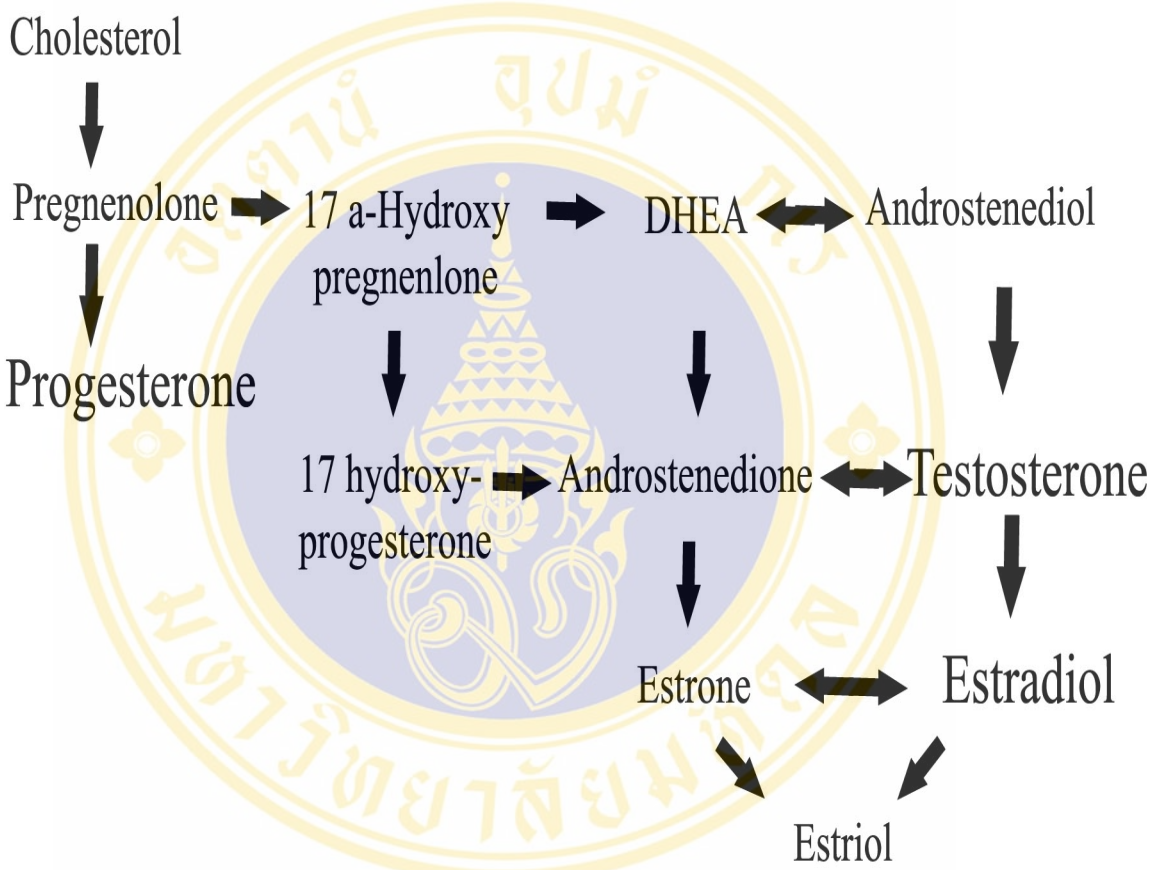


Table 4. Quantity of sex steroid hormone of various teleosts during spawning season.

Various teleosts	Hormone		
	Progesterone	Testosterone	11-ketotestosterone
Golden rabbitfish (Saydur et al., 2000)	7.98±2.12 ng/ml	17.35±3.63 ng/ml	4.38±0.59 ng/ml
Eurasian perch (Sulistyo et al, 2000)	-	6.8±2.4 ng/ml	1.3±0.4 ng/ml
Japanese huchen (Amer et al., 2001)	7.5 ng/ml	-	54.5±11.7 ng/ml
Pacific herring (Koya et al., 2002)	3.8±0.35 ng/ml	103.5±32.7 pg/ml	<0.1 ng/ml
Spotted halibut (Koya et al., 2003)	<0.2 ng/ml	-	1.3±0.4 ng/ml
Black rockfish (Mori et al., 2003)	0.30±0.04 ng/ml	1.0±0.3 ng/ml	2.8±1.4 ng/ml
Florida gar (Orlando et al., 2003)	-	4.64 ng/ml	12.00 ng/ml

- = no data reported.

Watanabe et al. (1999) concluded that the brood pouch of pipefishes plays important roles in gas exchange, osmoregulation, ion-transport and embryo protection.

CHAPTER 4

MATERIALS AND METHODS

1. Serum and Organ Sampling

1.1 Seahorse capture and experimental site

Adult brooding and non-brooding males *H. kuda* were collected in the sea near Ang-Sila, Chonburi province, by skin diving at 2-6 m depth. About 20 seahorses were caught in each month of the reproductive season (from December 2002 to March 2003).

1.2 Serum sampling

Prior to blood drawal and organ dissection, seahorse was individually weighed and measured. About 1 ml blood was drawn from the caudal arteries of each seahorse with a heparinized syringe. The serum was separated by centrifugation at 4000 rpm, 4°C for 20 min, and then stored at –20°C until analysis for the sex steroid contents.

1.3 Organ sampling

The testes were dissected and individually weighed and measured.

2. Histology

2.1 Testicular histology

The testes were dissected and processed immediately for light microscopic study (Figure 4). They were fixed in Bouin's fixative for 24 h, washed in 70% ethanol to remove the Bouin's fixative, dehydrated in graded ethanol series (70% ethanol → absolute ethanol), cleared in xylene and embedded in paraplast for histological examination. The tissues were serially sectioned at 5-7 μm thickness, and stained with hematoxylin & eosin for general morphology observation. The specimens were observed under a bright field microscope (Olympus BX51) and photographs were taken with an Olympus DP50 digital camera (Figure 5).

2.2 Brood pouch histology

Pieces of dissected brood pouch were processed immediately for light microscopic study. They were fixed in Bouin's fixative for 24 h, washed in 70% ethanol to remove the Bouin's fixative, dehydrated in graded ethanol series (70% ethanol → absolute ethanol), cleared in xylene and embedded in paraplast for histological examination. The tissues were serially sectioned at 5-7 μm thickness, and stained with hematoxylin & Masson trichrome for general morphology observation. The specimens were observed under a bright field microscope (Olympus BX51) and photographs were taken by an Olympus DP50 digital camera.

3. Steroid Immunoassay

3.1 Estradiol

Analysis of estradiol was obtained by the Roache Elecsys Estradiol II (Neogen corporation). A 35 μl plasma was incubated with estraidiol-specific biotinylated antibody in an assay cup (1st incubation) for 5 min. Streptavidin-coated microparticles and an estradiol derivative labeled with a ruthenium complex were added into an immunocomplex form (1st incubation) for 5 min. The entire complex became bound to the solid phase via interaction of biotin and streptavidin and then the reaction mixture was aspirated into the measuring cell. The microparticles were magnetically captured onto the surface of the electrode. Unbound substances were removed with procell. Application of a voltage to the electrode then induced chemiluminescent emission. These were measured by a photomultiplier. Results were determined via a calibration curve. This curve was instrument-specifically generated by 2-point calibration and a master curve (Figure 6).

3.2 Progesterone

Analysis of progesterone was obtained with Immulite Progesterone Analyzer (Neogen corporation). A 50 μl plasma was pipetted into a cup of labeled unit containing one bead coated with polyclonal rabbit antiprogesterone. A ligand-labeled synthetic progesterone and an alkaline phosphatase conjugate to anti-ligand in buffer were added into the cup. An immunocomplex form was incubated and then a cup of an immunocomplex was washed by a clean kit. A chemiluminescent substrate was

added into the cup that was incubated for 5 min. The cup was measured by a photomultiplier. Results were determined via a calibration curve as mention previously (Figure 7).

3.3 Testosterone

Analysis of testosterone was obtained with Immulite Testosterone Analyzer (Neogen corporation). A 20 µl plasma was pipetted into a cup of labeled unit containing one bead coated with polyclonal rabbit antitestosterone. A ligand-labeled synthetic testosterone and an alkaline phosphatase conjugated to anti-ligand in buffer were added into the cup. An immunocomplex form was incubated for 5 min and then a cup of an immunocomplex was washed by a clean kit. A chemiluminescent substrate was added into the cup which was incubated for 5 min. The cup was measured by a photomultiplier. Results were determined via a calibration curve as mention previously (Figure 8).

4. Statistical Analyses

4.1 Percentage of spermatogenic cell types

Percentage of a particular spermatogenic cell type was determined from randomly selected 5 testis sections of brooding or non-brooding testes in each month. The number of each particular spermatogenic cell type was counted in randomly selected areas of each slide. The number of a particular spematogenic cell type over the total number of cells was then determined. The average percentage of each cell type was calculated from 5 different sections.

4.2 Statistical test

Average and standard deviation (SD) were calculated to describe data of weight (mm), length (cm) and spermatogenic cell type (percentage). All these data were tested for normality using Shapiro-Wilk Test ($P<0.05$). Two-sample T test was used to compare body and testis characteristics of brooding and non-brooding males, and their spermatogenic cell types. All data were analyzed using Statistix v. 7 software for Windows.

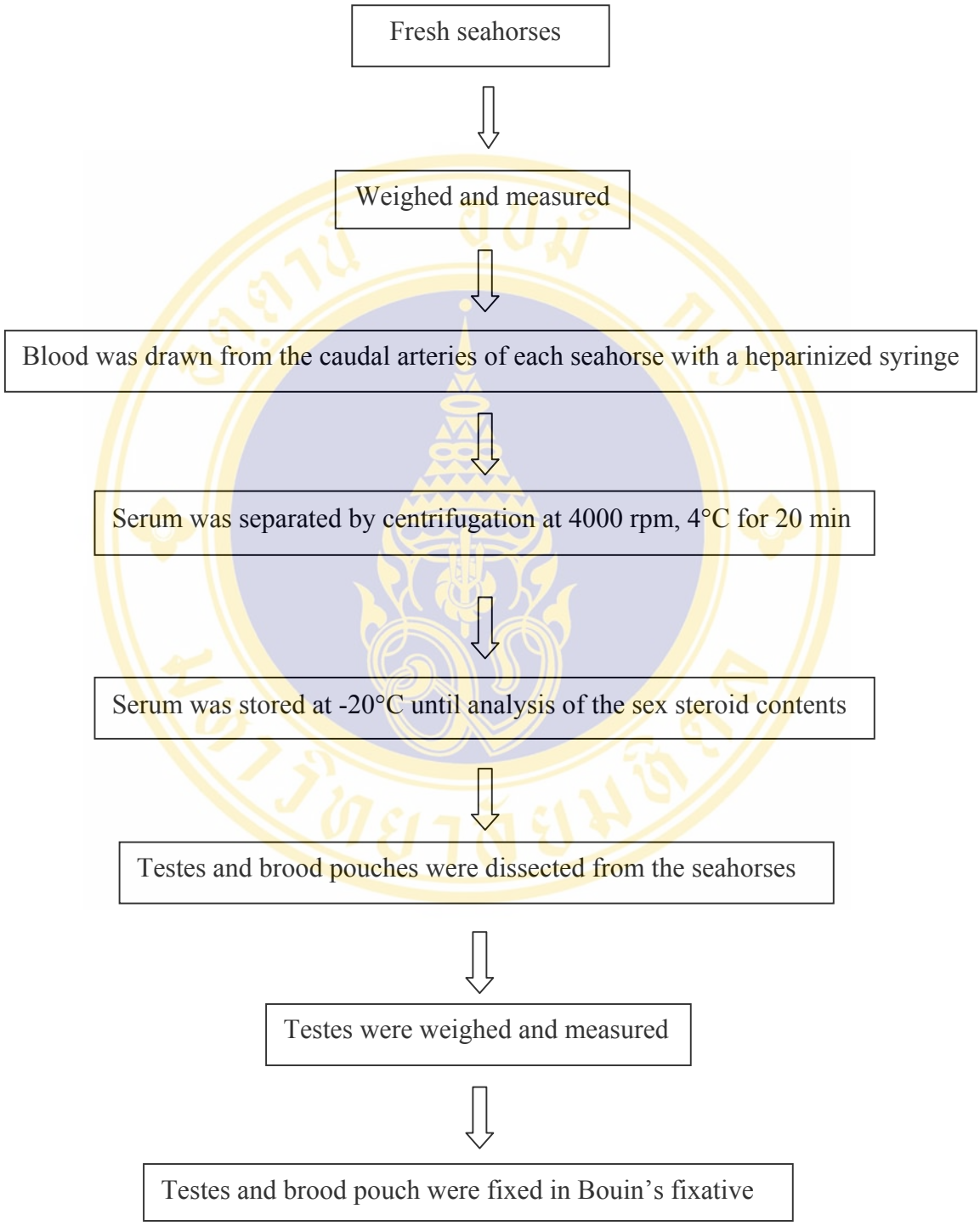


Figure 4. Protocol of serum and organ sampling.

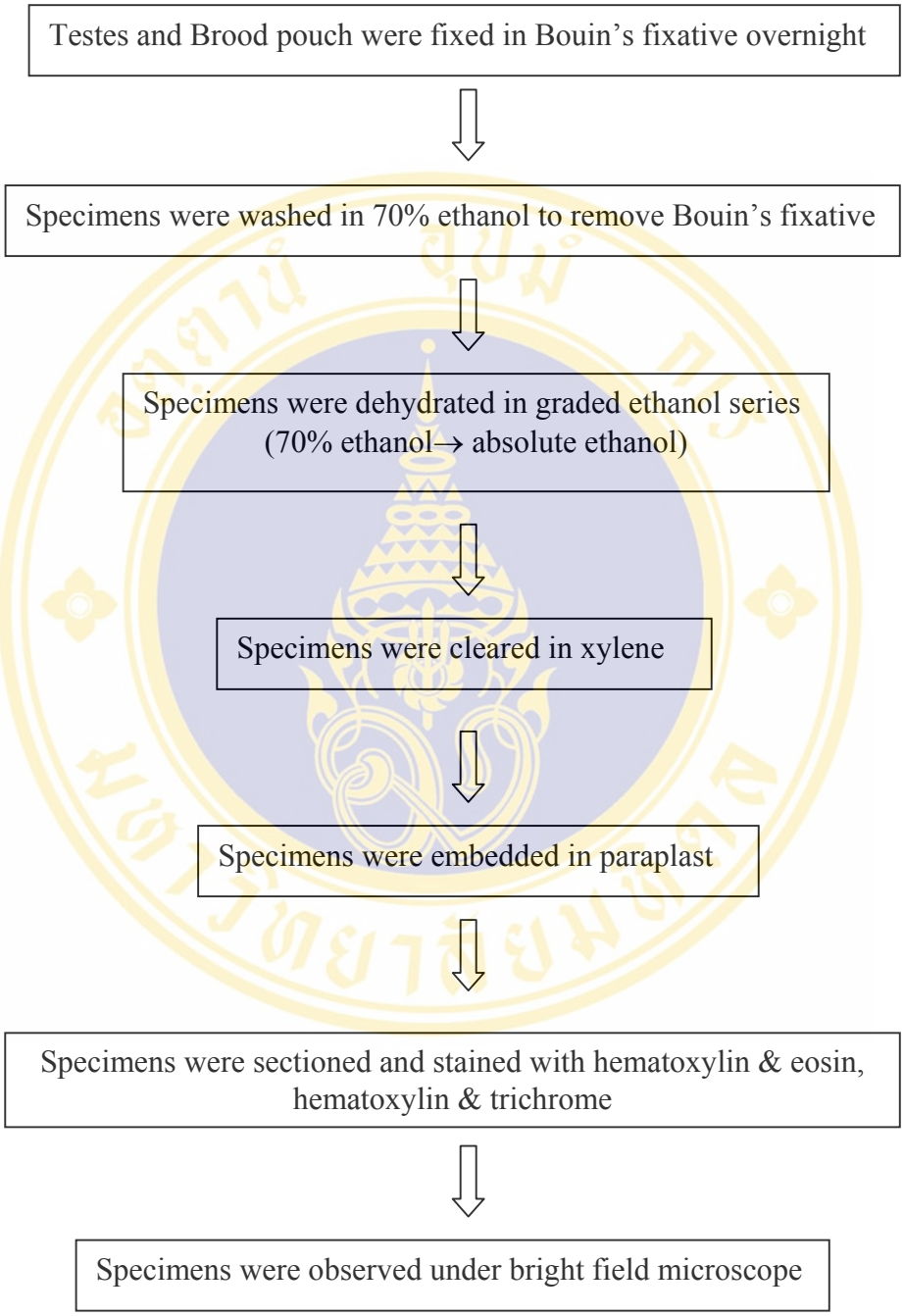


Figure 5. Protocol of the tissue preparation for light microscopy.

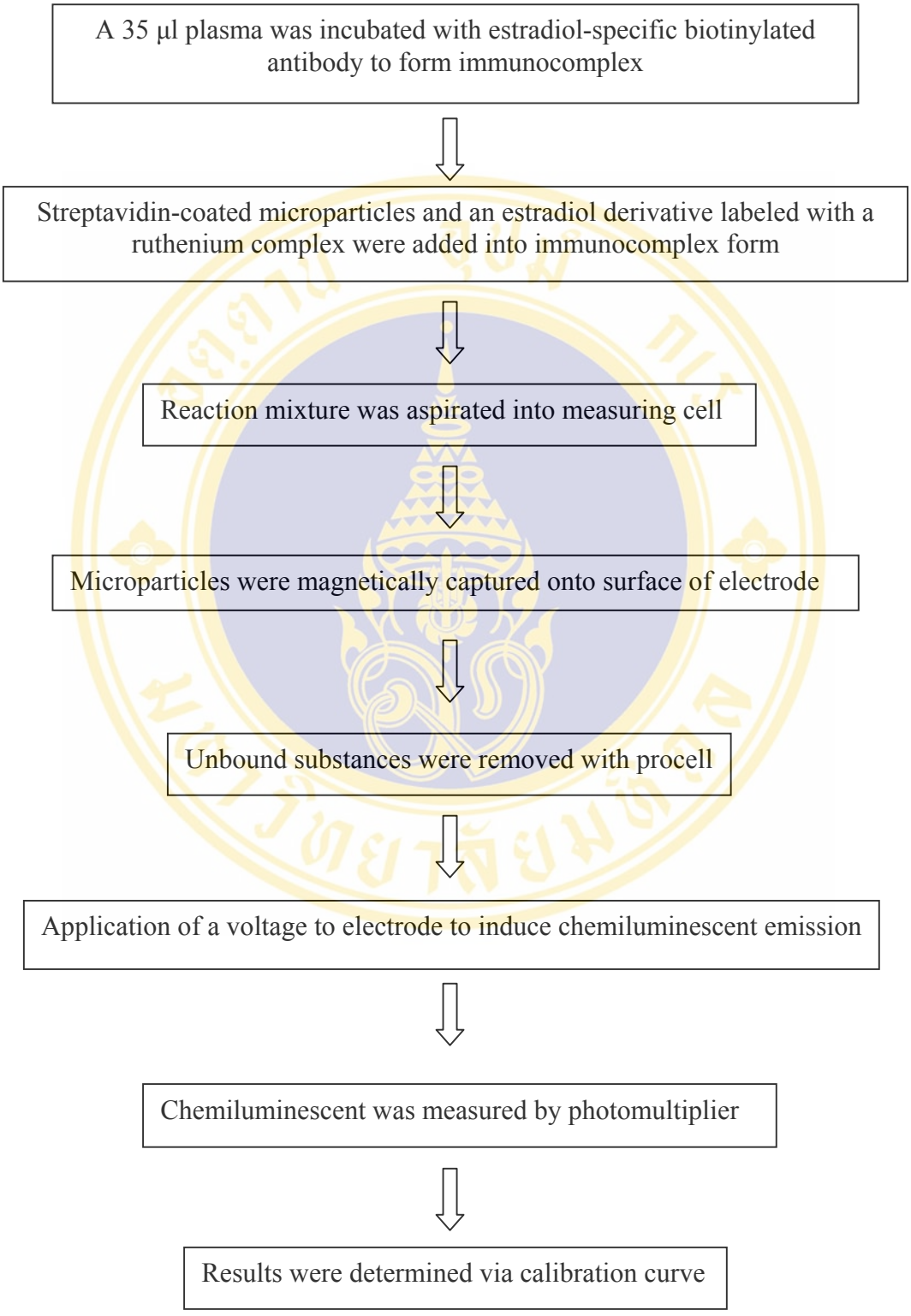


Figure 6. Protocol of estradiol content analysis.

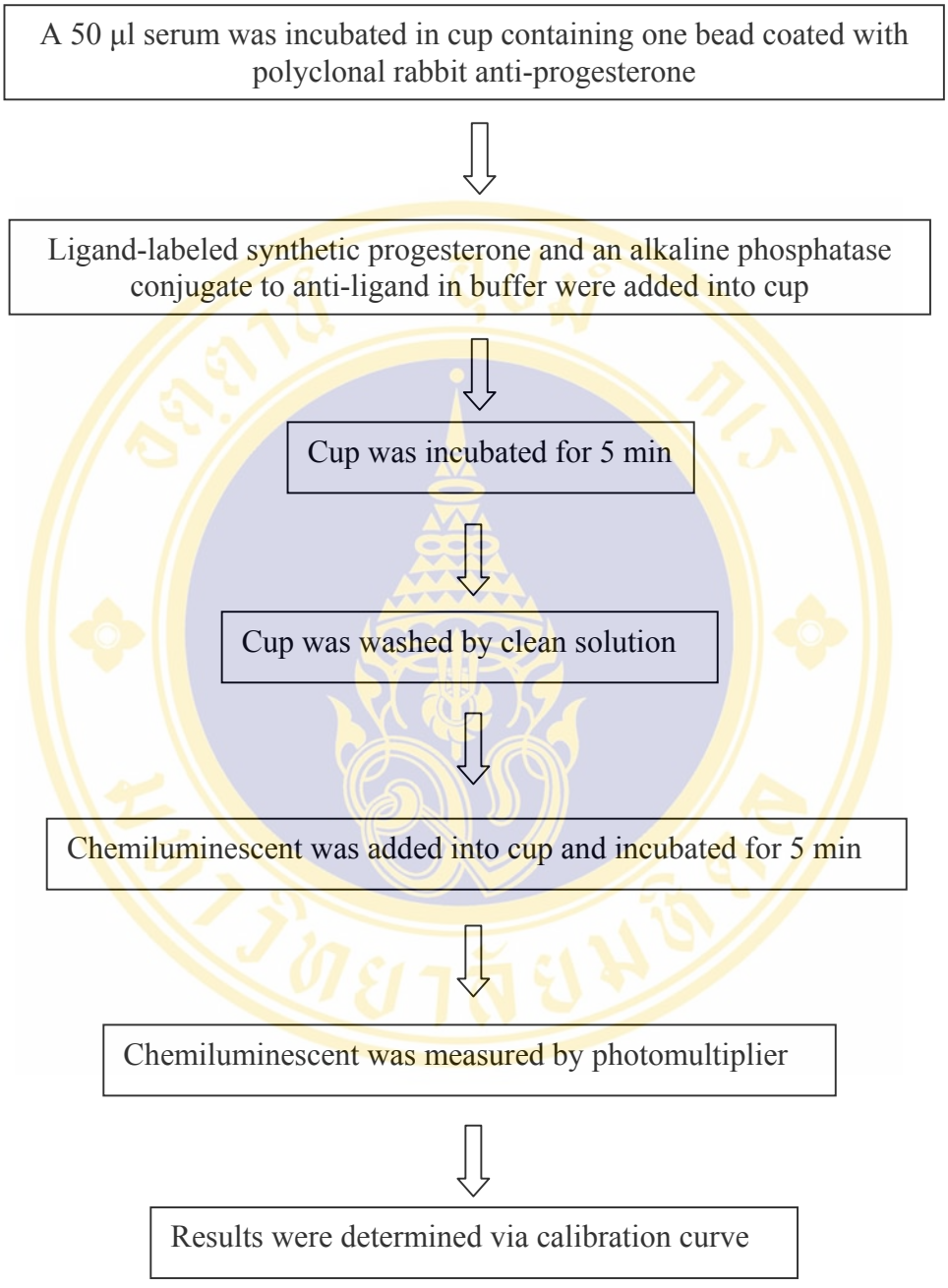


Figure 7. Protocol of progesterone content analysis.

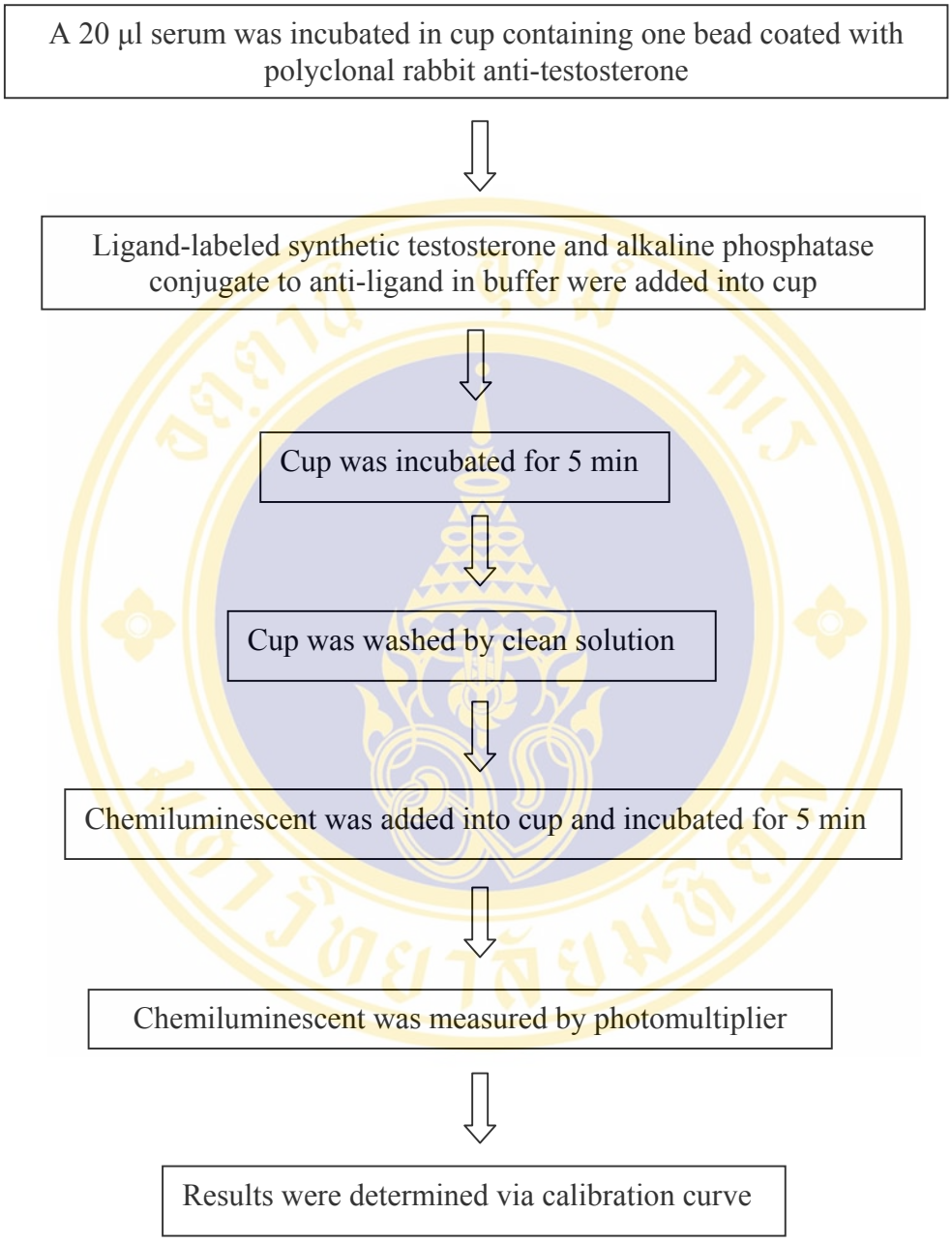


Figure 8. Protocol of testosterone content analysis.

CHAPTER 5

RESULTS

1. Body and Testis Length and Weight

The morphology of non-brooding and brooding males is shown in Figures 9A and 9B. The brooding males had higher average weight (14.21±4.15 g) than the non-brooding males (9.68±4.11 g) since they carried embryos in the brood pouch. But in term of body length, the brooding and the non-brooding males were about the same size (14.04±1.70 cm and 13.28±2.30 cm, respectively). The testis length and the weight of the brooding and the non-brooding males were not significantly different (Table 5). The average paired-testis weight of the brooding males (0.038±0.02 g) was higher than that of the non-brooding males (0.034±0.02 g). The testis length of the brooding males (1.13±0.30 cm) was longer than that of the non-brooding males (1.06±0.36 cm). At each month (during December to March), the body and testis length were not significantly different while the testis weight of the non-brooding males in December (0.06±0.02 g) was significantly different from those from the other months (Table 6).

2. Testicular Morphology and Histology

2.1 Testicular morphology

The testicular morphology of brooding and non-brooding males was similar. The testes were situated in the posterior of the body cavity, ventral to the kidney (Figure 10A). The testes of non-brooding (Figure 10B) and brooding males (Figure 10C) were paired, elongated and semi-translucent sausage-like organs. They were encapsulated by the external vascularized tunica albuginea enveloping a germinal epithelium and basement membrane. The tunica albuginea epithelium contained several blood vessels. The testes of brooding (Figure 11A) and non-brooding (Figure 12A) males

Figure 9. Non-brooding (A) and brooding (B) males, *H. kuda*.

Table 5. Average length and weight of the body and the testis of brooding and non-brooding males.

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Measurement	P	Brooding male	Non-brooding male	Total
Body length (cm)	0.1559 (NS)	14.04±1.70 N=29	13.28±2.30 N=26	13.68±2.02 N=55
Body weight (g)	0.0002*	14.21±4.15 N=29	9.68±4.41 N=26	12.07±4.81 N=55
Testis length (cm)	0.4909 (NS)	1.13±0.30 N=29	1.06±0.36 N=26	1.10±0.33 N=55
Testis weight (g)	0.4990 (NS)	0.038±0.02 N=29	0.034±0.02 N=26	0.036±0.02 N=55

NS = not significantly different ($P>0.05$).

* = significantly different ($P<0.05$).

Table 6. Average length and weight of the body and the testis of brooding and non-brooding males in each month.

Body weight and length of brooding and non-brooding males			
	Month	Brooding males	Non-brooding males
Body length (cm)	Dec 02	13.90±2.40	10.92±4.38
	Jan 03	14.10±1.49	13.92±2.19
	Feb 03	14.00±1.68	13.23±1.78
	Mar 03	14.20±0.95	13.90±1.74
	Average	14.04±1.70	13.27±2.27
Body weight (g)	Dec 02	13.41±3.81	8.29±4.83
	Jan 03	13.43±2.28	11.11±3.50
	Feb 03	13.38±5.23	10.57±4.52
	Mar 03	16.78±3.97	9.70±4.91
	Average	14.21±4.15	9.68±4.41
Testis length (cm)	Dec 02	1.29±0.28	1.06±0.43
	Jan 03	1.17±0.46	1.34±0.13
	Feb 03	1.03±7.31	1.05±0.40
	Mar 03	1.00±0.17	0.80±0.23
	Average	1.06±0.29	1.14±0.35
Testis weight (g)	Dec 02	0.059±0.015*	0.045±0.026
	Jan 03	0.039±0.025	0.036±0.016
	Feb 03	0.023±0.007	0.025±0.010
	Mar 03	0.023±0.017	0.022±0.016
	Average	0.038±0.022	0.034±0.020

* = significantly different from the other months ($P<0.05$).

Figure 10. A) Seahorse testes (arrow) are situated in the posterior of the body cavity, ventral to the kidney (K); B) Paired testes of non-brooding males; C) Paired testes of brooding males.

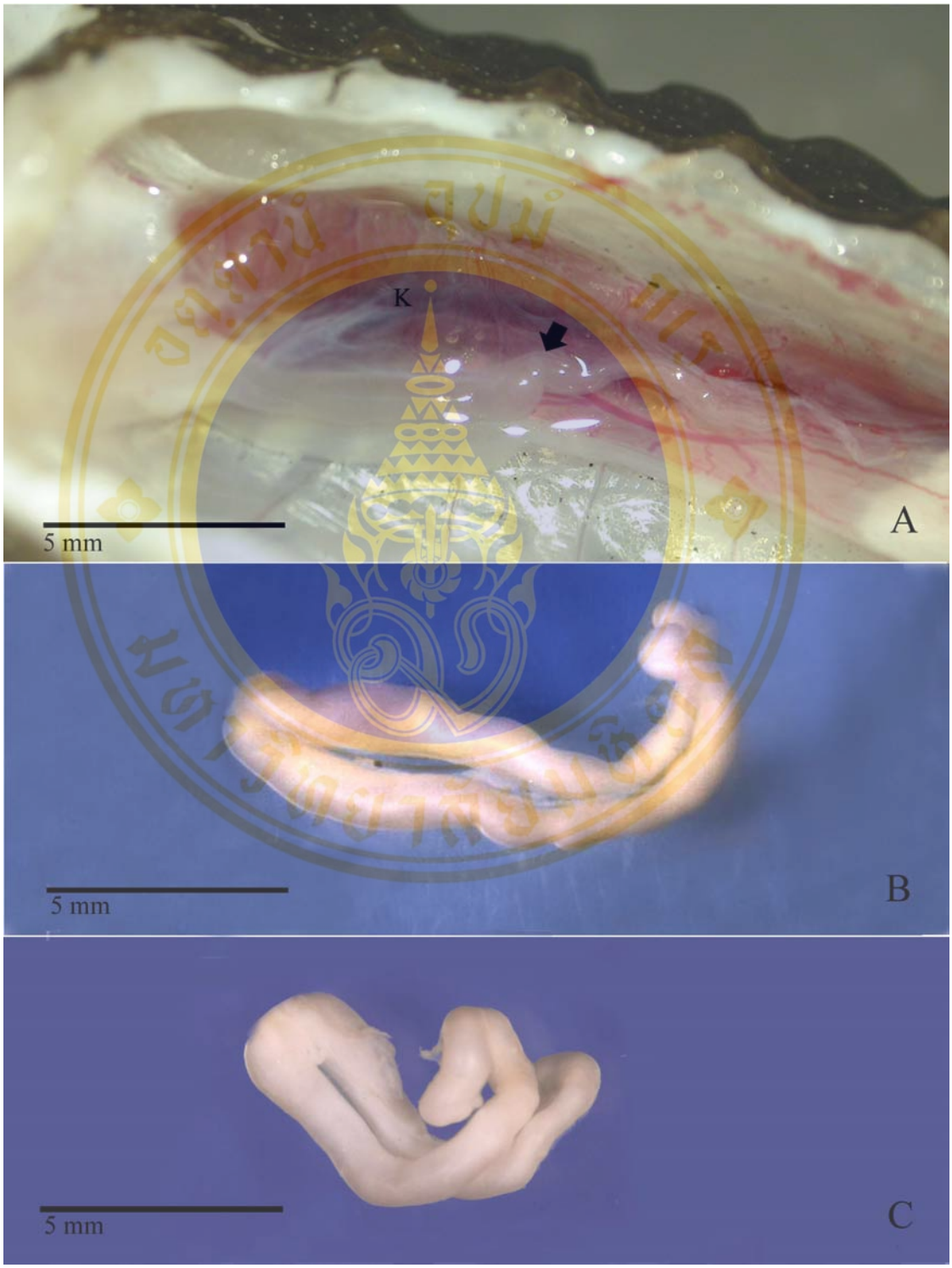
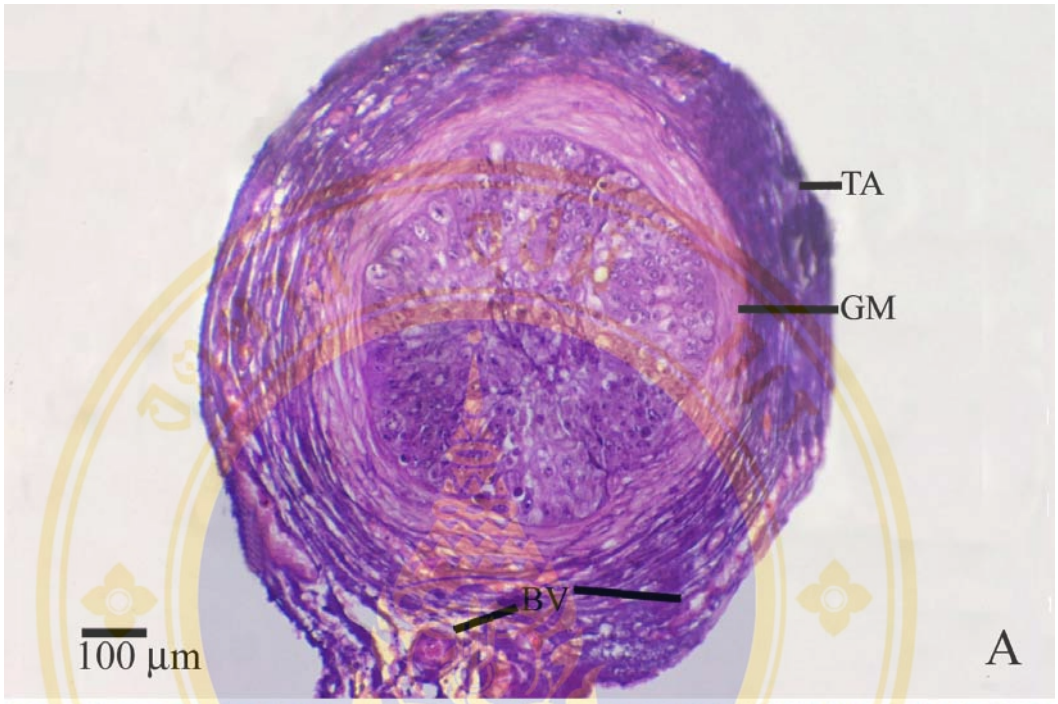


Figure 11. A) Brooding male testis consists of 2 epithelial layers: tunica albuginea epithelium (TA) and germinal epithelium (GM). Blood vessels (BV) are found in the tunica albuginea epithelium; B) Brooding male testis contains spermatogonia (Sg) and different stages of spermatocytes: primary (PrSc) and secondary spermatocytes (SSc).



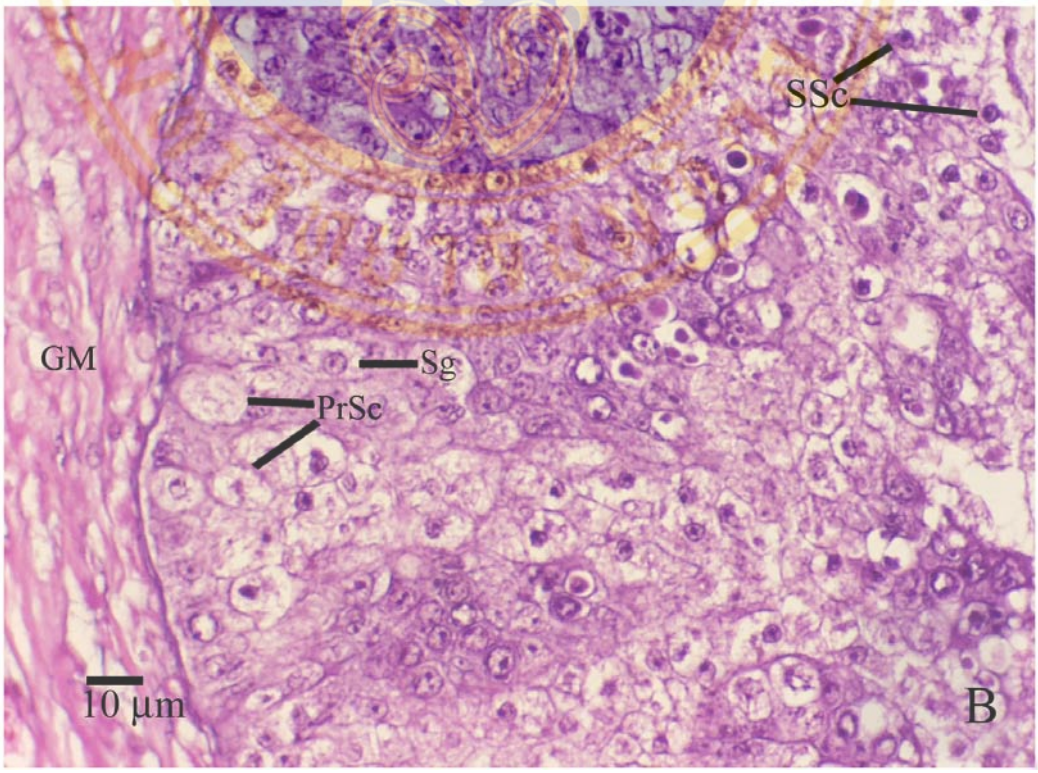
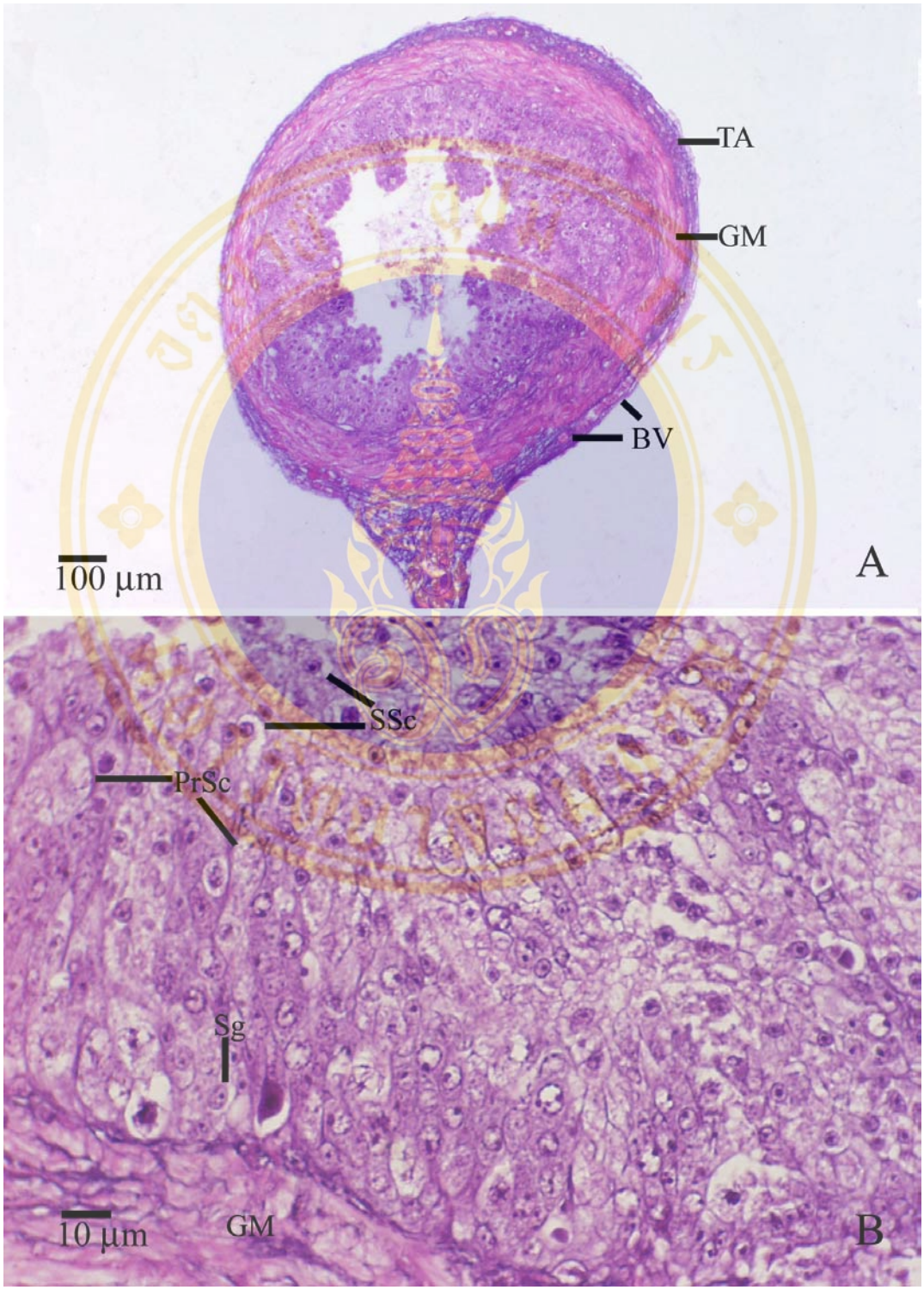


Figure 12. A) Non-brooding male testis consists of 2 epithelial layers: tunica albuginea epithelium (TA) and germinal epithelium (GM). Blood vessels (BV) are found in the tunica albuginea epithelium; B) Brooding male testis contains spermatogonia (Sg) and different stages of spermatocytes: primary (PrSc) and secondary spermatocytes (SSc).



appeared as tube containing spermatogonial cells and sperm-producing spermatocytes with a central lumen.

2.2 Testicular histology

2.2.1 Brooding male

During reproductive season (December to March), each testis of the brooding males contained spermatogonia and different stages of spermatocytes (Figure 11B). Various stages of spermatogenic cells observed in the testes were spermatogonia, 7 stages of primary spermatocytes, secondary spermatocytes and spermatids. Spermatogonia and primary spermatocytes were found along the entire length of the testes. Secondary spermatocytes and spermatids were discovered in the testis lumen but spermatozoa were not observed in the present study.

Spermatogonia (Sg)

Spermatogonia were supported by the basement membrane. They were large round-shaped with 8.05±0.79 μm in diameter, each with a distinct nucleus. The nucleus was spherical with 5.26±0.38 μm in diameter and contained lightly stained chromatin, and 1-3 prominent nucleoli (Figure 13A).

Primary spermatocyte (PrSc)

Primary spermatocytes were differentiated from spermatogonia. The cell size of 11.15±1.18 μm in diameter containing darkly stained spherical nucleus was observed. Various stages of the primary spermatocytes were identified by patterns of chromatin organization. Stages of the primary spermatocytes found in the present study were:

1. Leptotene spermatocyte (LSc)

The cell was spherical and contained a round nucleus with nucleolus. Some chromatin fibers were thin. A small amount of chromatin was observed as a thin rim and scattered along the nuclear membrane (Figure 13B).

2. Zygotene spermatocyte (ZSc)

The cell shape was similar to the leptotene spermatocyte. Within the nucleus, some chromatin fibers increased in size and density (Figure 13B).

3. Pachytene spermatocyte (PSc)

The cell was spherical. Within the nucleus, long fibers of the paired chromatins were seen (Figure 13C).

4. Diplotene spermatocyte (DSc)

The cell shape was similar to the previous stage. The chromatin became increasingly condense and lie close together (Figure 13C).

5. Diakinesis spermatocyte (DiSc)

The chromatin was condensed and distributed in the center of the cell (Figure 13D).

6. Metaphase spermatocyte (MSc)

The nuclear membrane disappeared. The condensed chromatin plates were aligned at the equatorial region (Figure 14A).

7. Anaphase spermatocyte (ASc)

The condensed chromatin plates were separated to the opposite poles (Figure 14B).

Secondary spermatocyte (SSc)

With approximately 6.99±0.69 μm in diameter, the secondary spermatocytes were smaller than the primary ones. Each cell contained darkly stained spherical nucleus with 4.07±0.38 μm in diameter. The secondary spermatocytes were immersed in the fibrilar materials in the lumen (Figure 14C).

Spermatid (St)

The spermatids were the smallest cell with 3.6±0.42 μm in diameter. The cell was irregular in shape. The nucleus was 2.03±0.29 μm in diameter (Figure 14C-D).

2.2.2 Non-brooding male

Various stages of spermatogenic cells were found in the testes of the non-brooding males during December to March (Figure 12B). Their cell size and general characteristics of spermatogonia (Figure 15A), 7 stages of primary spermatocytes: leptotene (Figure 15B), zygotene (Figure 15C), pachytene (Figure 15D), diplotene (Figure 16A), diakinesis (Figure 16B), metaphase (Figure 16C) and anaphase spermatocytes (Figure 16D), secondary spermatocytes (Figure 17A-C) and spermatids (Figure 17B-D) were similar to those of the brooding males.

Figure 13. Various stages of spermatogenic cells in brooding male testis; A) Spermatogonia (Sg) are located on the basement membrane; B) Leptotene spermatocyte (LSc) shows nucleus contains nucleolus and thin chromatin fibers; zytotene spermatocyte (ZSc) shows some increase in size and density of the chromatin fibers; C) Pachytene spermatocyte (PSc) shows nucleus contains long dense chromatin contains condensed; diplotene spermatocytes (DSc) shows chromatin blocks; D) Diakinesis spermatocytes (DiSc) shows condensed chromatin blocks distributed in the center of the cell.

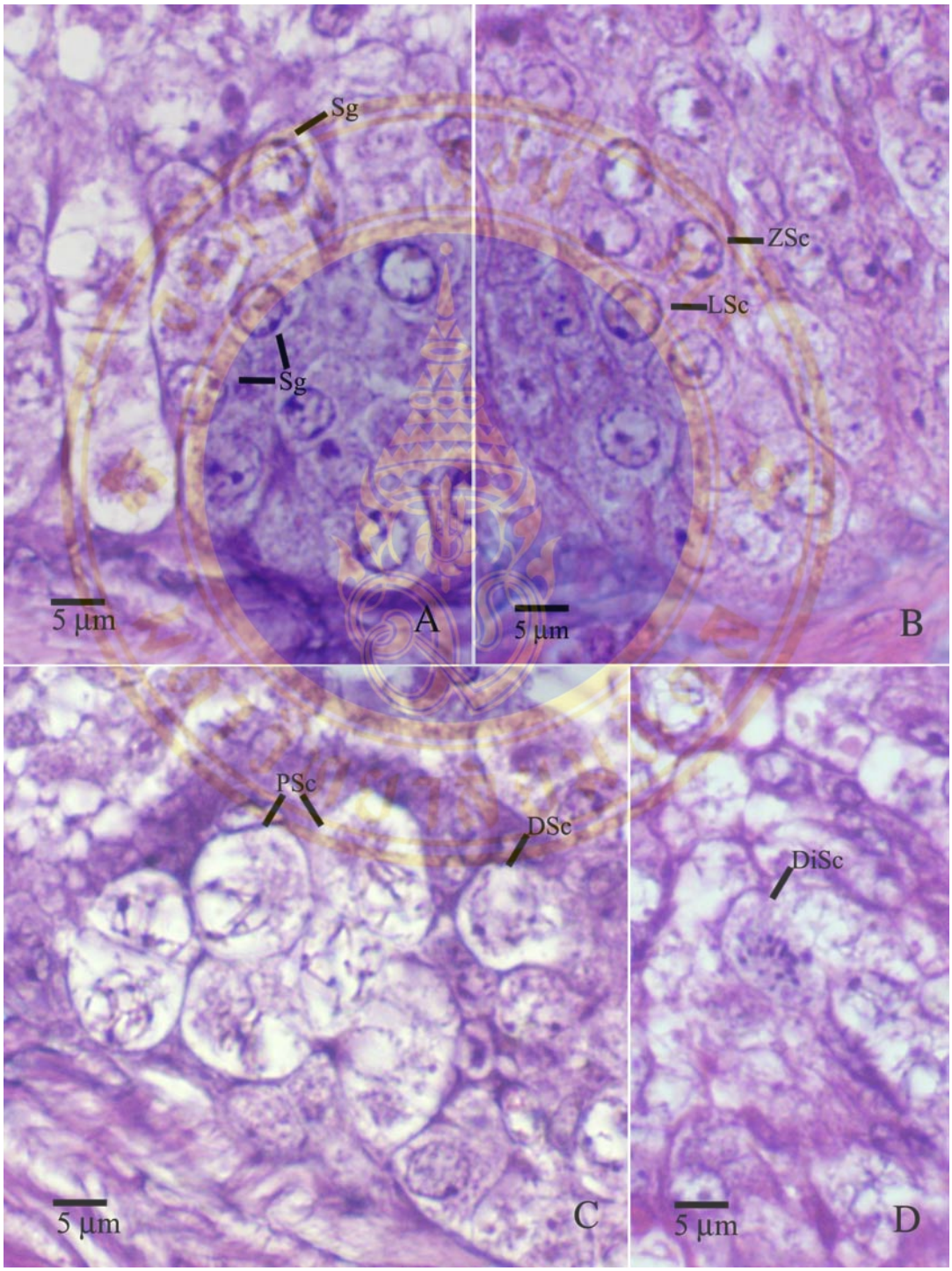


Figure 14. Various stages of spermatogenic cells in brooding male testis; A) Metaphase spermatocyte (MSc) displays condensed chromatin plates aligned at equatorial region; B) Anaphase spermatocyte (ASc) shows separation of the condensed chromatin plates to the opposite poles; C) Secondary spermatocyte (SSc) and spermatid (St) are found in testis lumen; D, E) Spermatids (St) are irregular in shape.

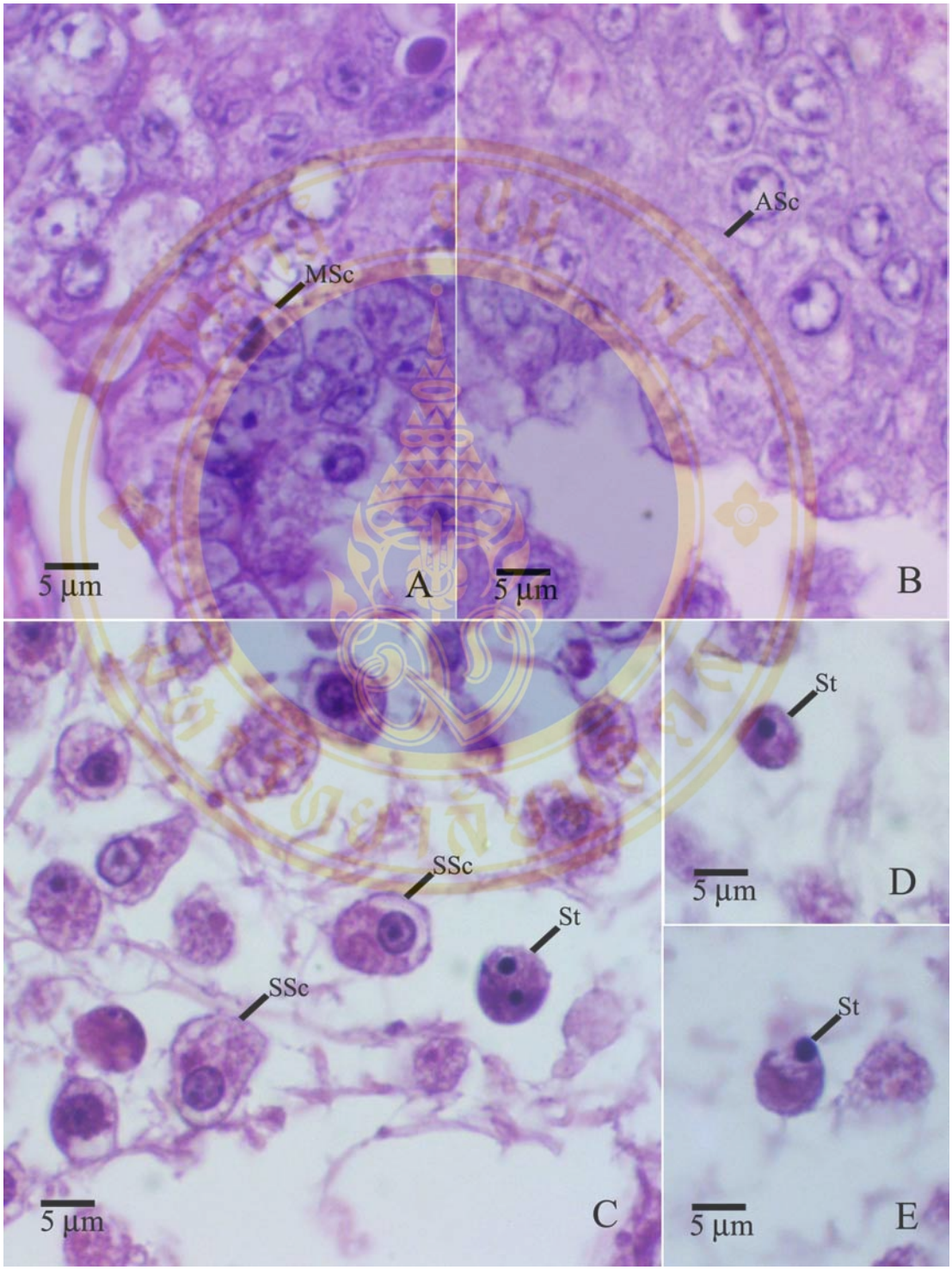


Figure 15. Various stages of spermatogenic cells in non-brooding male testis; A) Sermatogonia (Sg) are located on the basement membrane; B) Leptotene spermatocyte (LSc) shows nucleus contains nucleolus and thin chromatin fibers; C) Zygotene spermatocyte (ZSc) shows some increase in size and density of the chromatin fibers; D) Pachytene spermatocyte (PSc) shows nucleus contains long dense chromatin contains condensed.

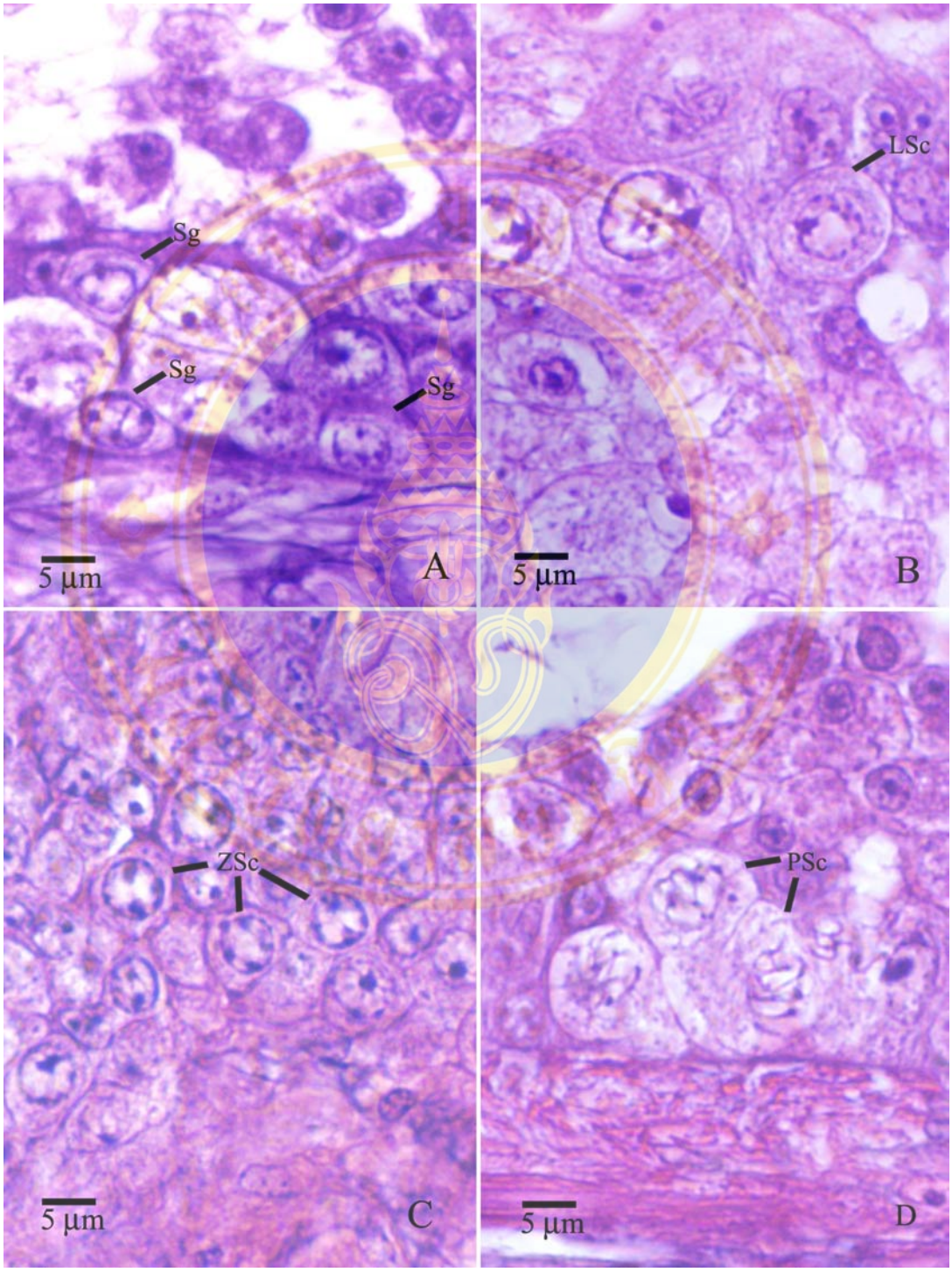


Figure 16. Various stages of spermatogenic cells in non-brooding male testis; A) Early diplotene spermatocyte (DSc) shows chromatin blocks; B) Diakinesis spermatocyte (DiSc) shows condensed chromatin blocks distributed in the center of the cell; C) Metaphase spermatocyte (MSc) shows condensed chromatin plates aligned at the equatorial region; D) Anaphase spermatocyte (ASc): separation of the condensed chromatin plates to the opposite poles.

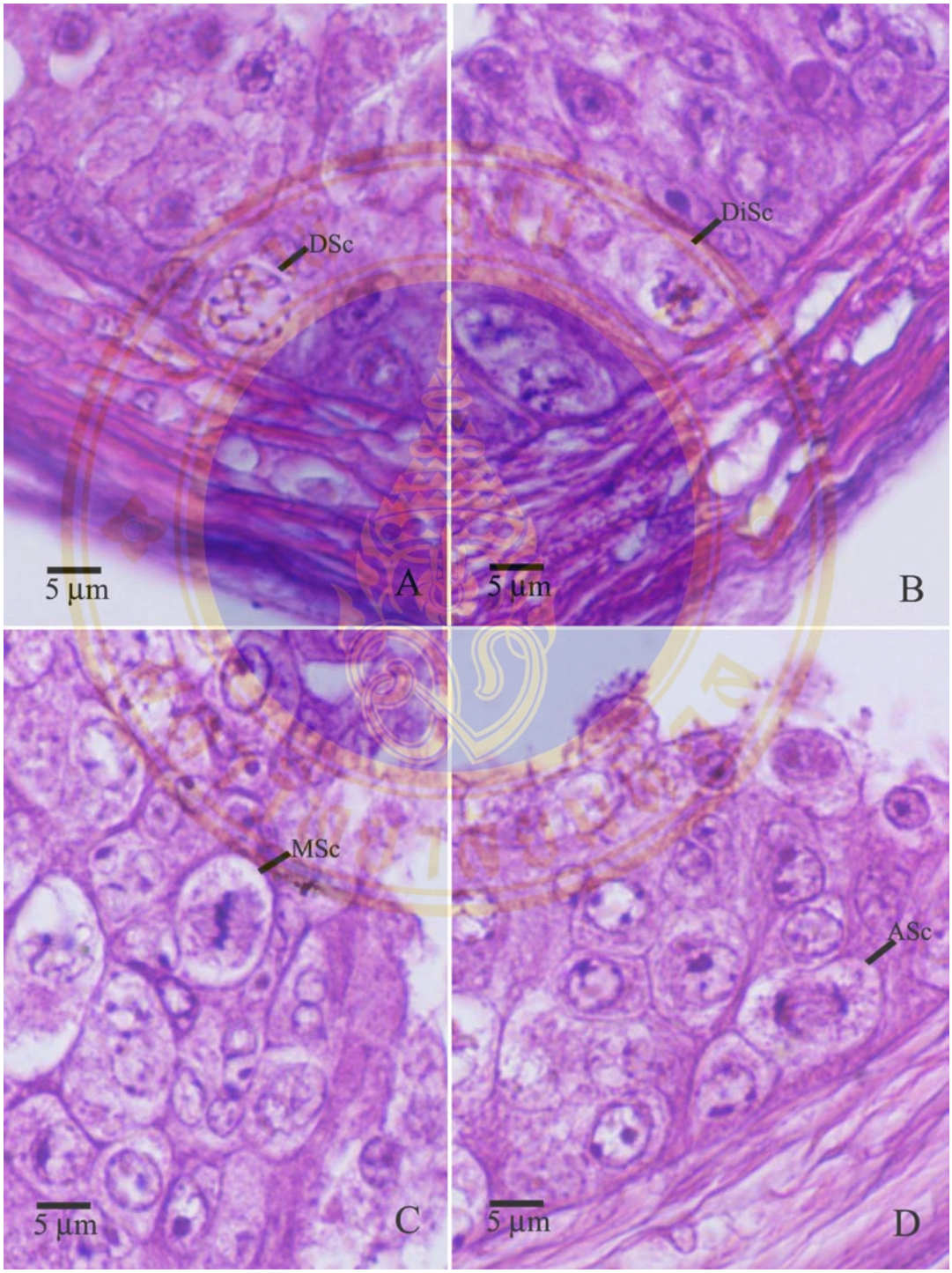
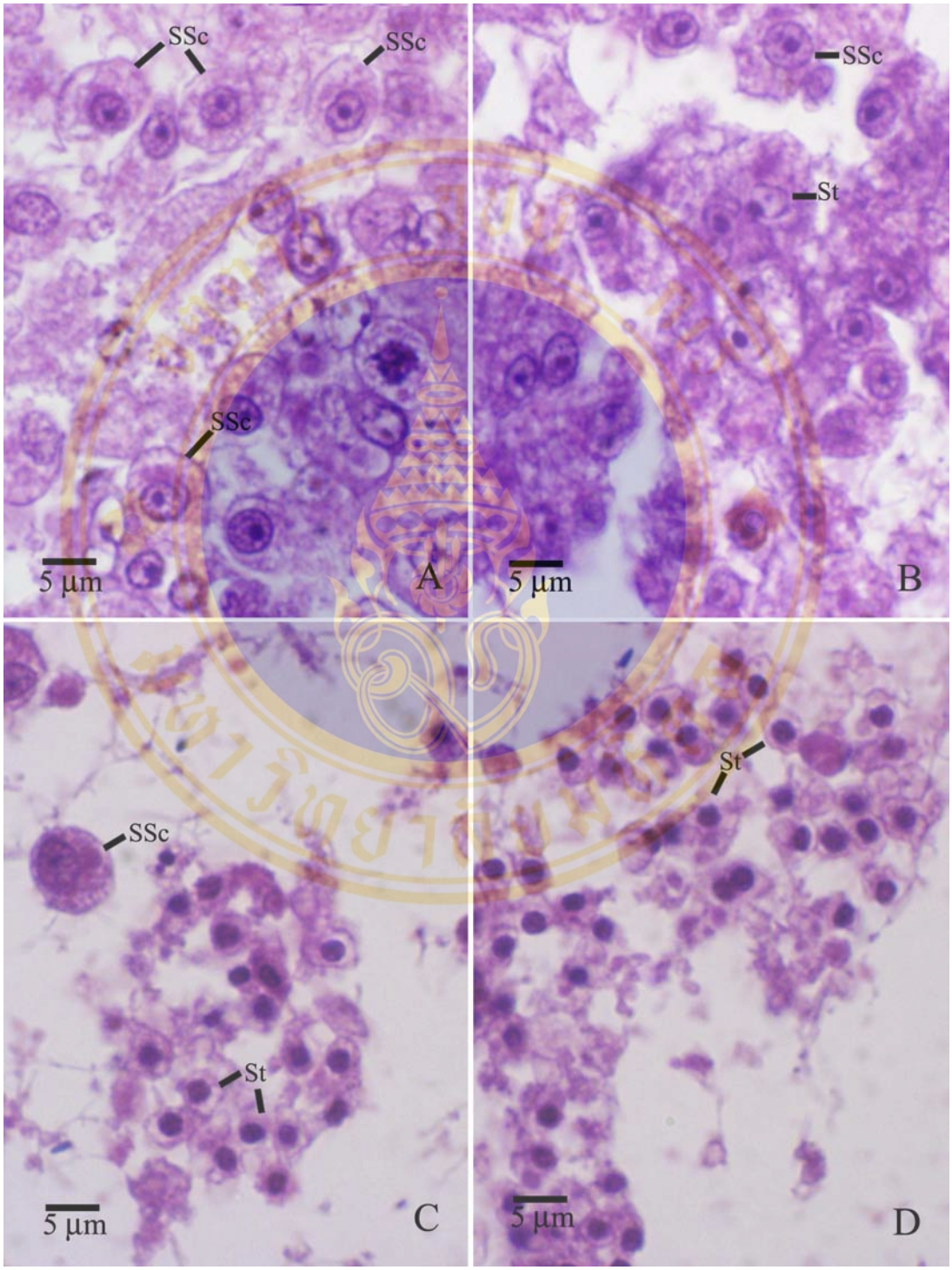


Figure 17. Various stages of spermatogenic cells in non-brooding male testis; A) Secondary spermatocytes (SSc) are immersed in the fibrilar material; B, C) Secondary spermatocyte (SSc) and spermatid (St) found in the testicular lumen; D) Spermatid (St) is irregular in shape.



3. Percentage of Spermatogenic Cell Type

3.1 Brooding male

The percentage of various spermatogenic cells in the brooding males during December-March is shown in Figure 18. In the testes, secondary spermatocyte was found at the highest percentage followed by those of primary spermatocyte, spermatogonia and spermatid, respectively. During the reproductive season, the percentage of the spermatogonia was high in December and gradually decreased towards the end of the season while that of the primary spermatocyte was not different. In contrast, the percentage of the secondary spermatocyte was high in January to February. The percentage of spermatid gradually increased from December to March.

3.2 Non-brooding male

The percentage of the spermatogenic cells in the non-brooding males is shown in Figure 19. Similar to cells in the testis of the brooding males, the percentage of secondary speramtocyte was highest followed by those of primary spermatocyte, spermatogonia and spermatid, respectively. During the reproductive season, the percentage of spermatogonia in each month was similar. The percentage of primary spermatocyte was low in the mid-season (January-February) while that of the secondary spermatocyte gradually increased from December to February. The percentage of spermatid was high in January compared to those in December and March.

4. Steroid Immunoassay

During the reproductive season, the estradiol levels of the brooding and the non-brooding males in each month were not significantly different ($P>0.05$). During brooding period, the estradiol level was highest in December (67.6 ng/ml), then decreased to the lowest level in January (23.6 ng/ml) and gradually increased again in February (28.9 ng/ml) and March (49.9 ng/ml). In the non-brooding males, a peak of estradiol level was in December (58.2 ng/ml) and gradually dropped to the lowest level in February (23.1 ng/ml) before increasing in March (37.4 ng/ml) (Figure 20). Testosterone and progesterone levels of the brooding and the non-brooding males in each month were low (< 0.2 ng/ml).

5. Brood Pouch Morphology and Histology

5.1 Brood pouch morphology

Brood pouch of spotted seahorse was located between abdomen and tail. It is shaped like a pocket and has a small pore while opens anteriorly. The brood pouch was enlarged when carrying developing embryos (Figure 21A). Enlarged blood vessels were found in the brood pouch of the brooding male (Figure 21B). The brood pouch of the non-brooding male was flattened (Figure 21C) and had fewer number of blood vessels (Figure 21D).

5.2 Brood pouch histology

The brood pouch consisted of two layers of epithelium: the inner and the outer epithelia (Figure 22A). The inner layer was a pseudostratified epithelium (Figure 22B) while the outer layer (epidermis) was a stratified cuboidal epithelium (Figure 22C). A triangular structure was found in this layer. There were 3 layers of tissues between the outer and the inner epithelia. The inner layer was loose connective tissue (Figure 22D). The middle layer was smooth muscle (Figure 22E). The outer layer was dense irregular connective tissue (Figure 22F). The brood pouch consisted of 5 different stages (Figure 23 and Table 7):

5.2.1 Normal stage

The brood pouch of non-brooding male at normal stage had thick inner epithelium. The inner tissue layer was filled with loose connective tissue and appeared as a thick layer. Several small blood vessels appeared in all 3 layers but mostly found in the inner tissue layer. Smooth muscles were observed in the middle tissue layer (Figure 24A-E).

5.2.2 Embryo carrying stage

The brood pouch of brooding male with developing embryos attached to the inner tissue layer exhibited a decrease in surface area of the inner epithelium. Many blood vessels were distributed throughout all layers (inner, middle and outer). In the inner tissue layer, the blood vessels were highly distributed and had various sizes. Most large blood vessels were in the inner tissue layer while the small ones occurred near the inner epithelium particularly at the embryo-attaching areas. In the

middle and the outer tissue layers, the blood vessels had smaller size and less dense compared to those in the inner tissue layer (Figure 25A-E).

5.2.3 Embryo released stage

The stage at which the embryos had been released from the brood wall, the inner epithelium and the tissue layer were flattened and the surface formed a rupture surface appearance. Most blood vessels were enlarged and deposited throughout the inner tissue layer. Small blood vessels lied close to the inner epithelium. Smooth muscles were distributed in both the middle and outer tissue layers (Figure 26A-D).

5.2.4 Repair stage I

Brood pouch in the non-brooding male after the embryo were released, the inner epithelium and the tissue layer were at the repair stage. They became increase in size and were highly digitated. Many blood vessels of various sizes were distributed throughout the inner tissue layer while in the other layers (middle and outer tissue layers), small blood vessels were observed. Smooth muscles were also found in the middle and outer tissue layers (Figure 27A-D).

5.2.5 Repair stage II

At the repair stage II, the brood pouch wall became thickened. The inner epithelium was thick; the inner tissue layer was thick and filled with loose connective tissue. Blood vessels of various sizes were observed in the inner tissue layer; however, most of them became decrease in size. In the middle and the outer tissue layers, the small blood vessels and smooth muscles were randomly (Figure 28A-E).

Table 7 Different stages of the brood pouch in the brooding and non-brooding males.

Stage	Feature		
	Inner epithelium and tissue layer	Blood vessel	Distribution of smooth muscle
Non-brooding male			
Normal	Thick Filled with loose connective	Small size and highly distributed	In the middle tissue layer
Brooding male			
Embryo carrying	Thin Flattened and stretched	Various sizes and highly abundant Small size found near embryos	In the middle tissue layer
Embryo released	Thin and ruptured	Various sizes and highly abundant Small size found close to the embryos Large size found in the inner tissue layer	In the middle and outer tissue layers
Non-brooding male			
Repair I	Thick Highly digitated	Various sizes and highly abundant Small and large size found in the inner tissue layer	In the middle and outer tissue layers
Repairing II	Thick Filled with loose connective tissues	Various sizes and highly abundant Low density of large size	In the middle and the outer tissue layers

Figure 18. Percentage of different spermatogenic cells of brooding males in each month during reproductive season. Each plot represents mean±SD.

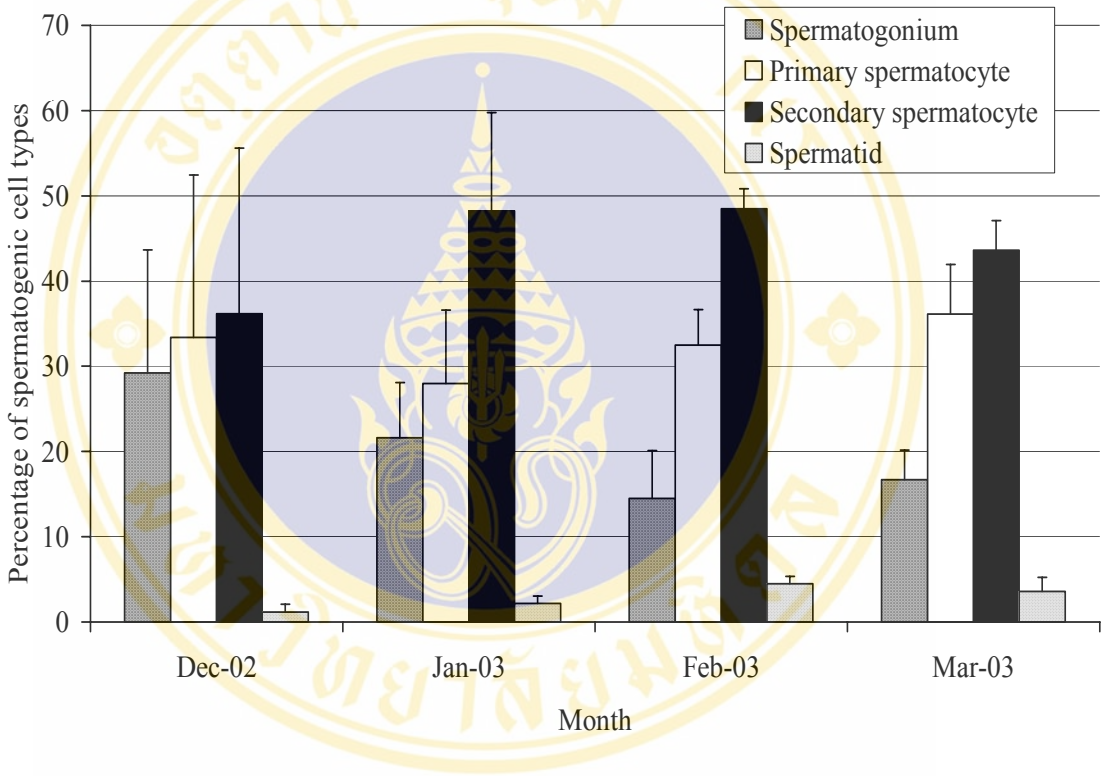


Figure 19. Percentage of different spermatogenic cells of non-brooding males in each month during reproductive season. Each plot represents mean±SD.

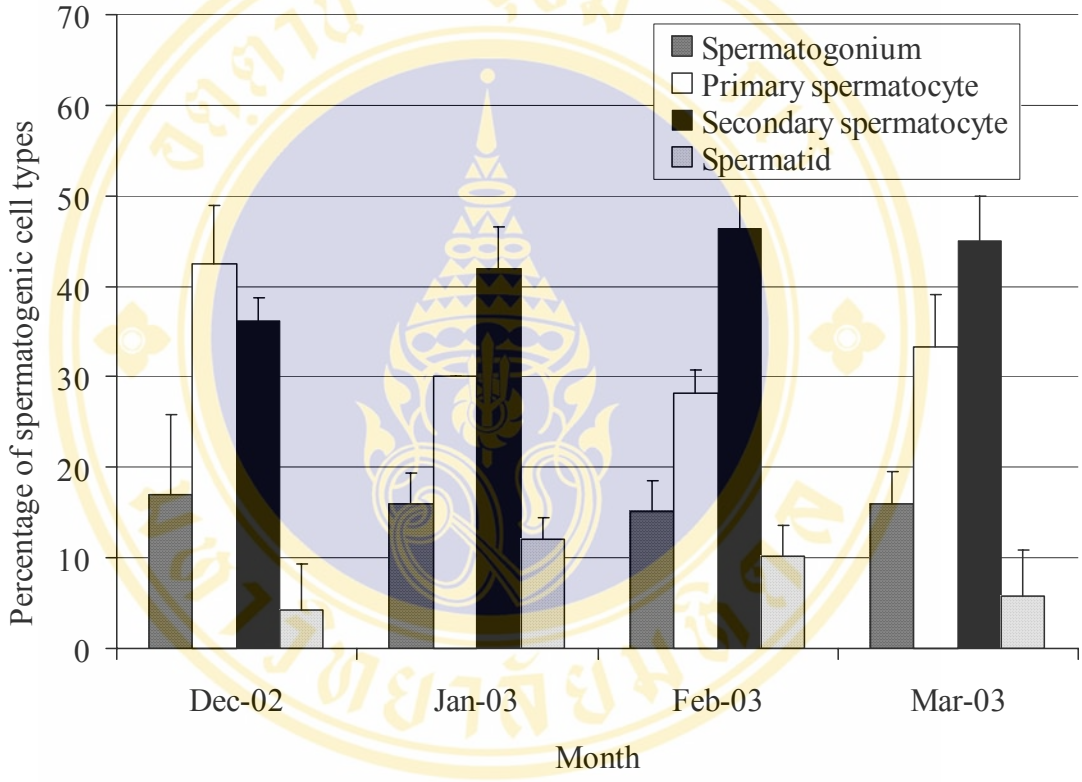


Figure 20. Estradiol levels in brooding and non-brooding males. Each plot represents serum pool from 6 samples.

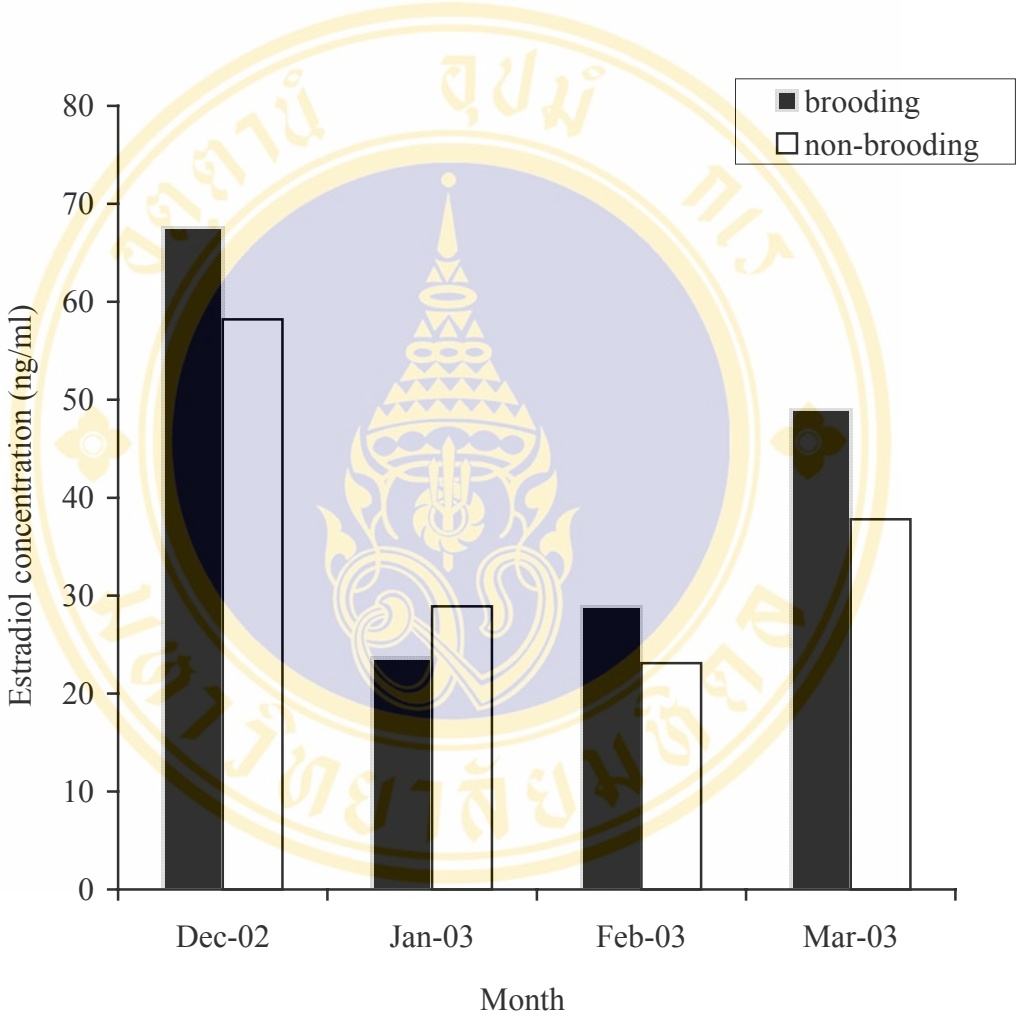


Figure 21. Brood pouch; A) Brood pouch of brooding males carrying embryos; B) Embryos were removed from brooding pouch; enlarged blood vessels are observed; C, D) Brood pouch of non-brooding males is flattened and shows fewer number of blood vessels.

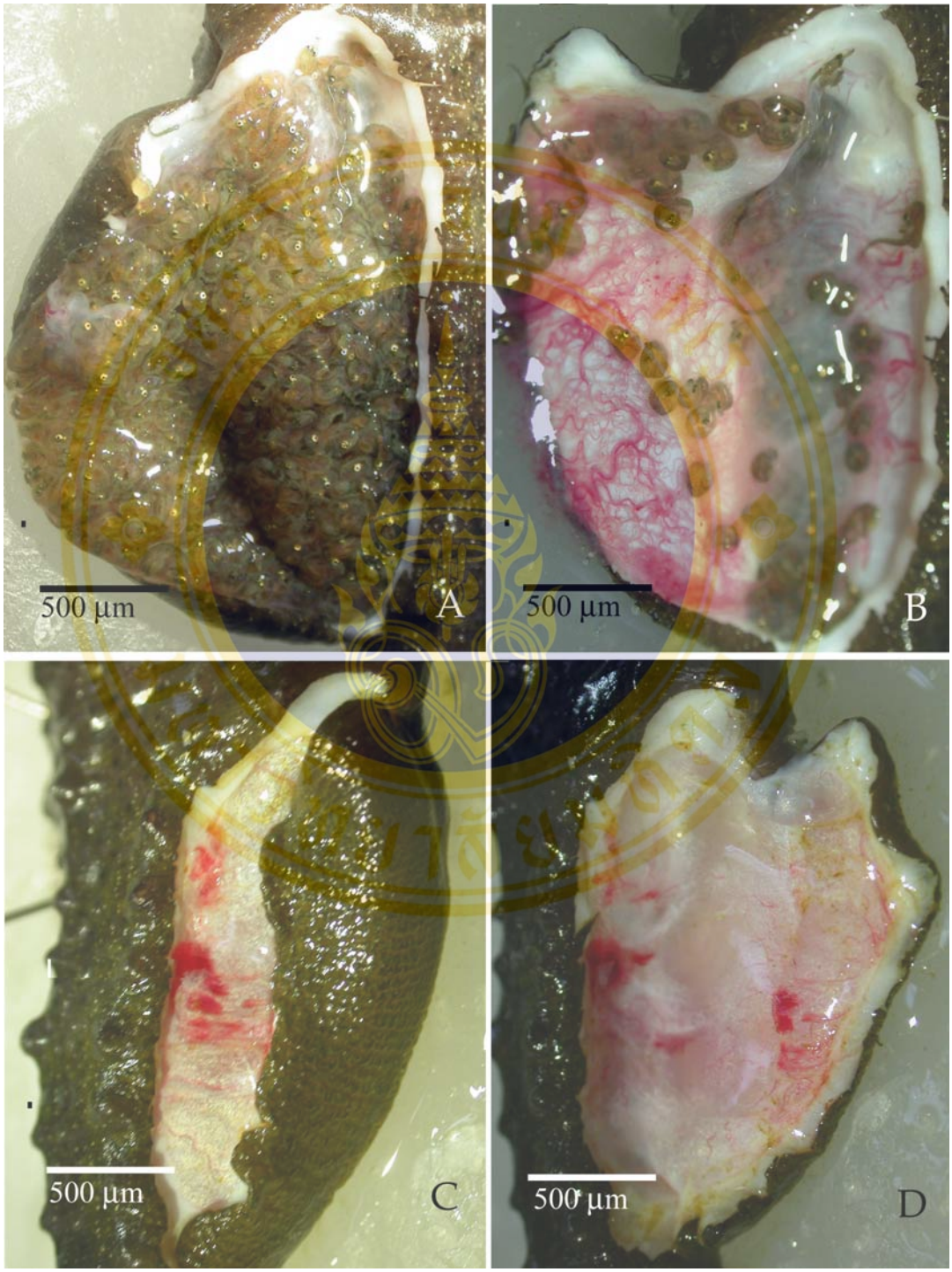


Figure 22. Histology of brood pouch in non-brooding male; A) Brood pouch consists of 2 epithelial layers: inner (IEP) and outer epithelia (OEP). There are 3 layers of tissues between outer and inner epithelia: outer (OL), middle (ML) and inner tissue layers (IL); B) Inner layer (IEP) is a pseudostratified epithelium; C) Outer layer (OEP) (epidermis) is stratified cuboidal epithelium with triangular giant structure (TGC); D) Inner tissue layer (IL) is loose connective tissues containing blood vessels (BV); E) Middle tissue layer (ML) contains smooth muscles with blood vessels (BV); F) Outer tissue layer (OL) is dense irregular connective tissue.

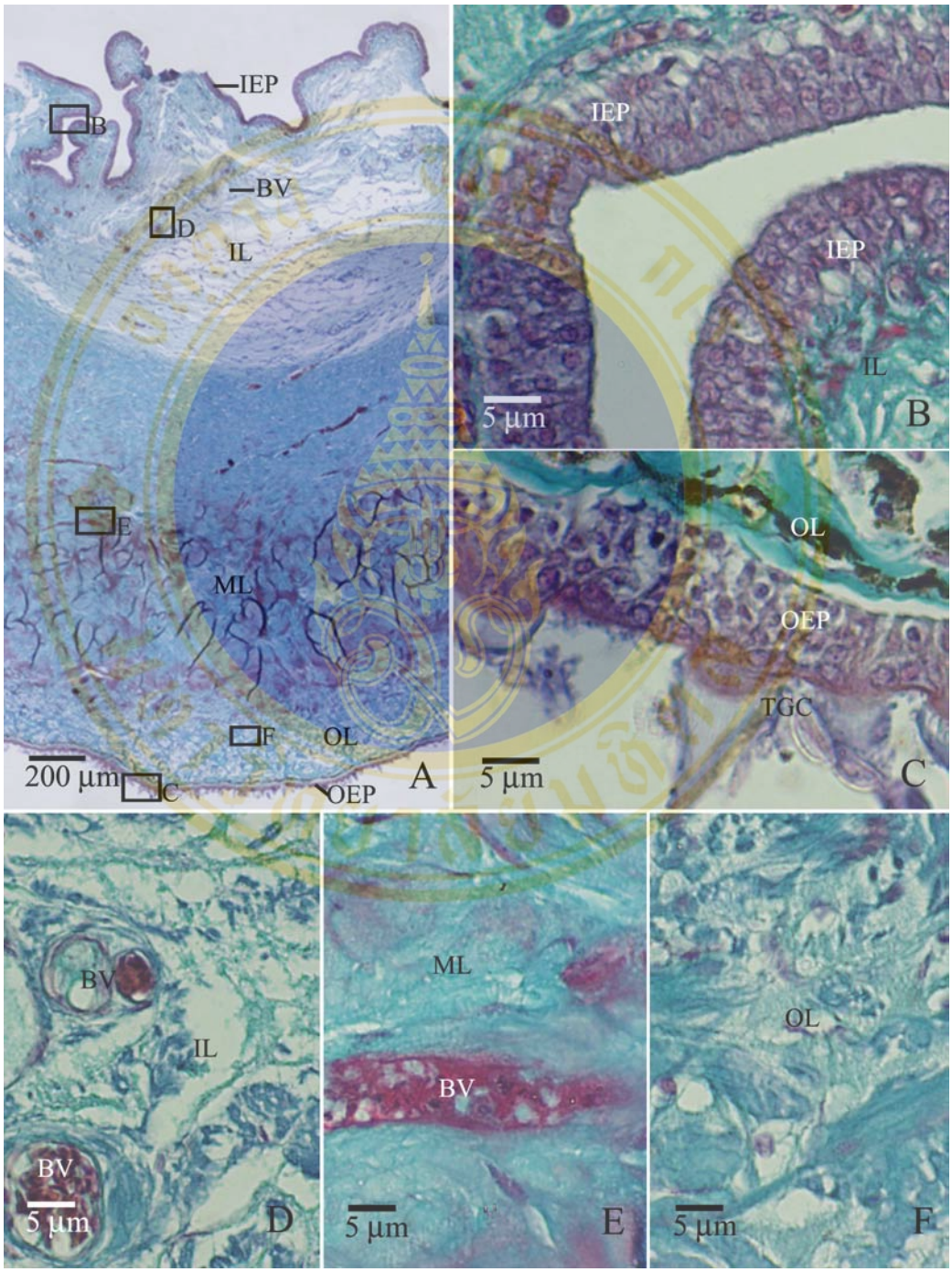


Figure 23. Sequential stages of brood pouch alteration; A) Normal stage in non-brooding male; B) Embryo carrying stage in brooding male; C) Embryo released stage in brooding male; D) Repair stage I in non-brooding male; E) Repair stage II in non-brooding male.

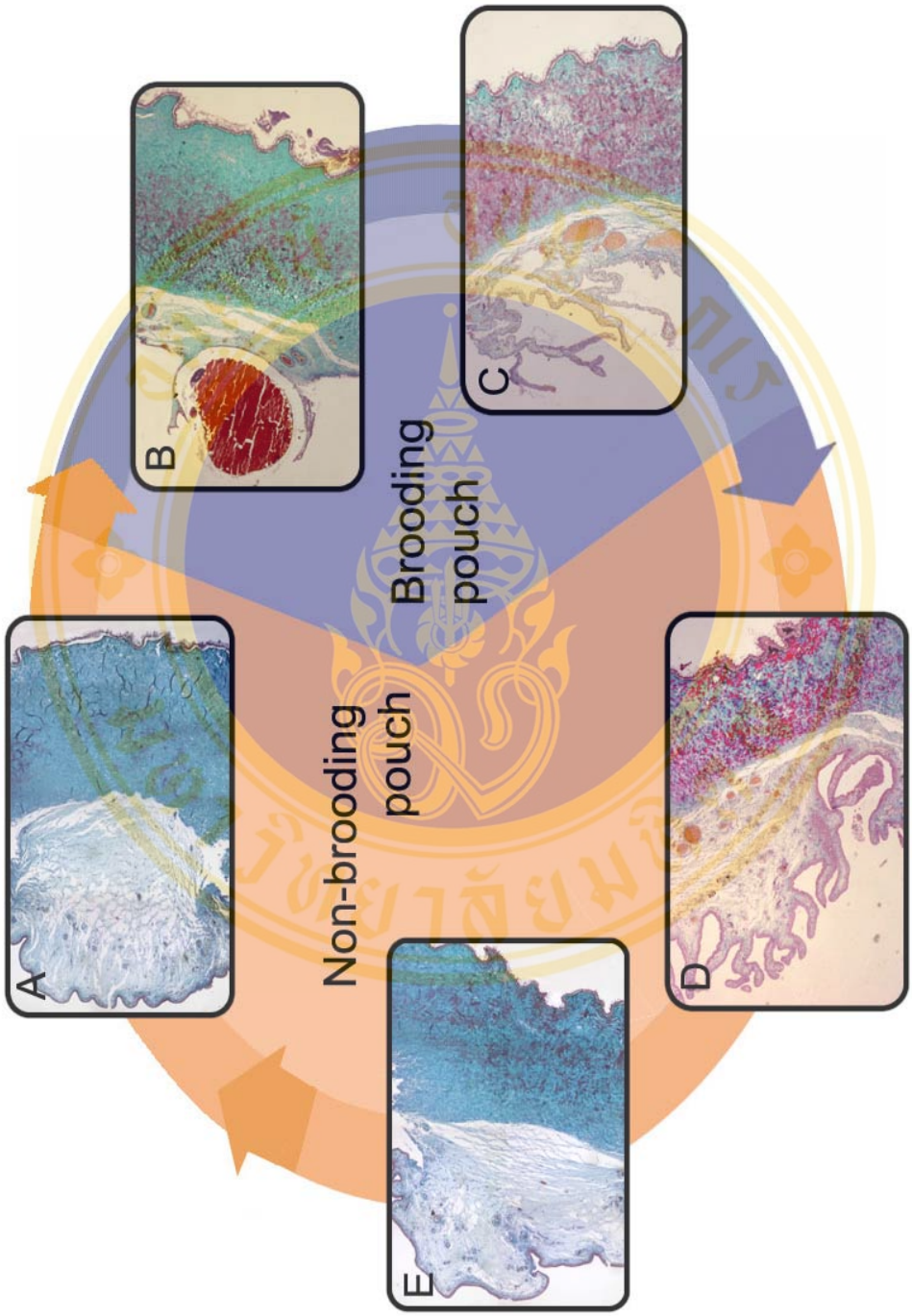


Figure 24. Normal stage brood pouch in non-brooding male; A) Inner epithelium (IEP) and inner tissue layer (IL) are thick; B) Epidermal cells are arranged in inner epithelium (IEP); C) Epidermal cells are arranged completely over inner tissue layer (IL), which are filled with loose connective tissue; D) Inner epithelium (IEP); E) Blood vessel (BV) in inner tissue layer (IL) became increase in size.

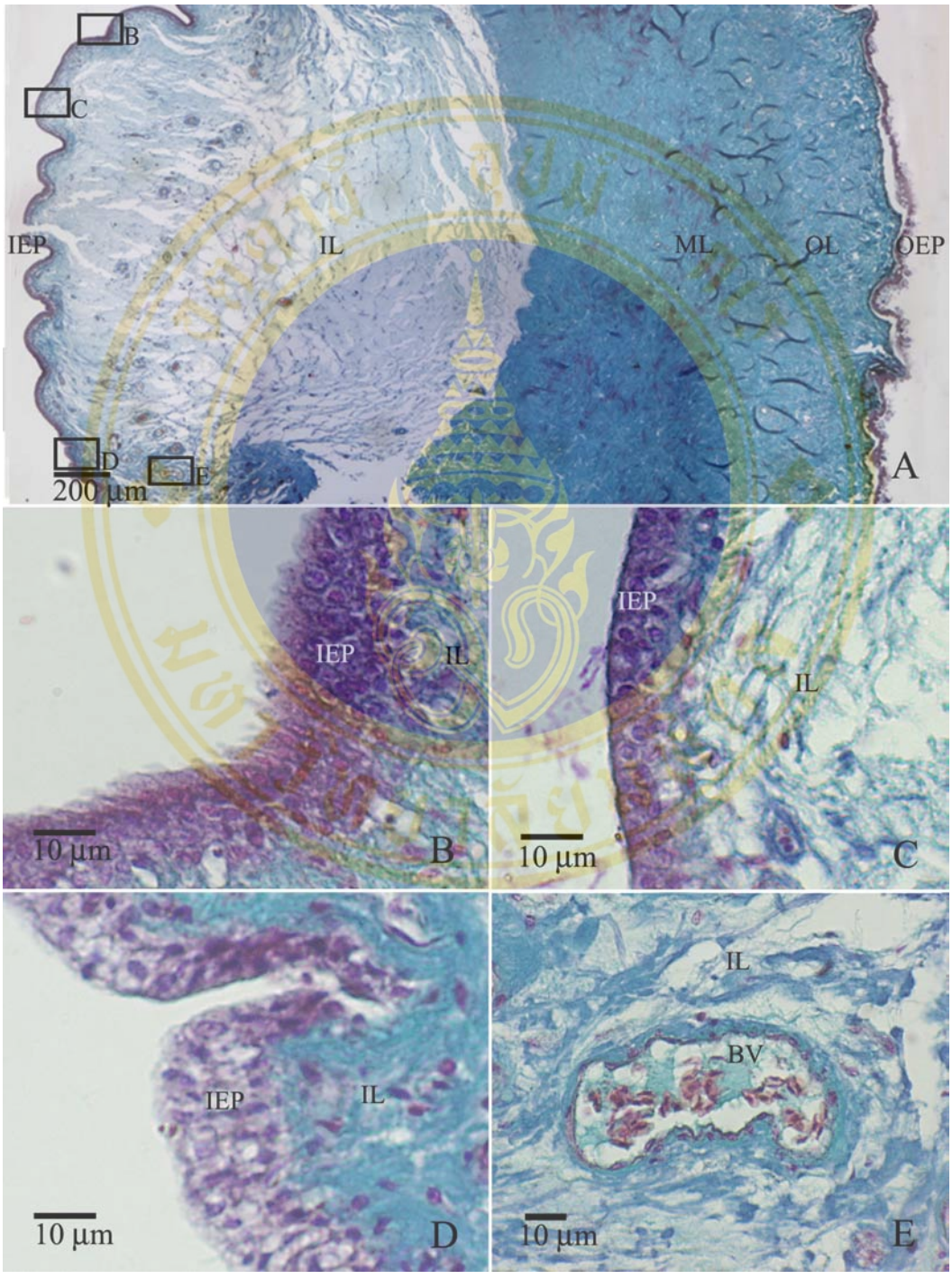


Figure 25. Embryo carrying stage in brooding male; A) Developing embryos attached to the inner tissue layer; B) Inner epithelium at the area close to embryo; C) Inner epithelium is flatten and stretched; D) Blood vessels (BV) is situated in the middle tissue layer (ML); E) Triangular giant structure (TGC) is found in the outer epithelium (OL).

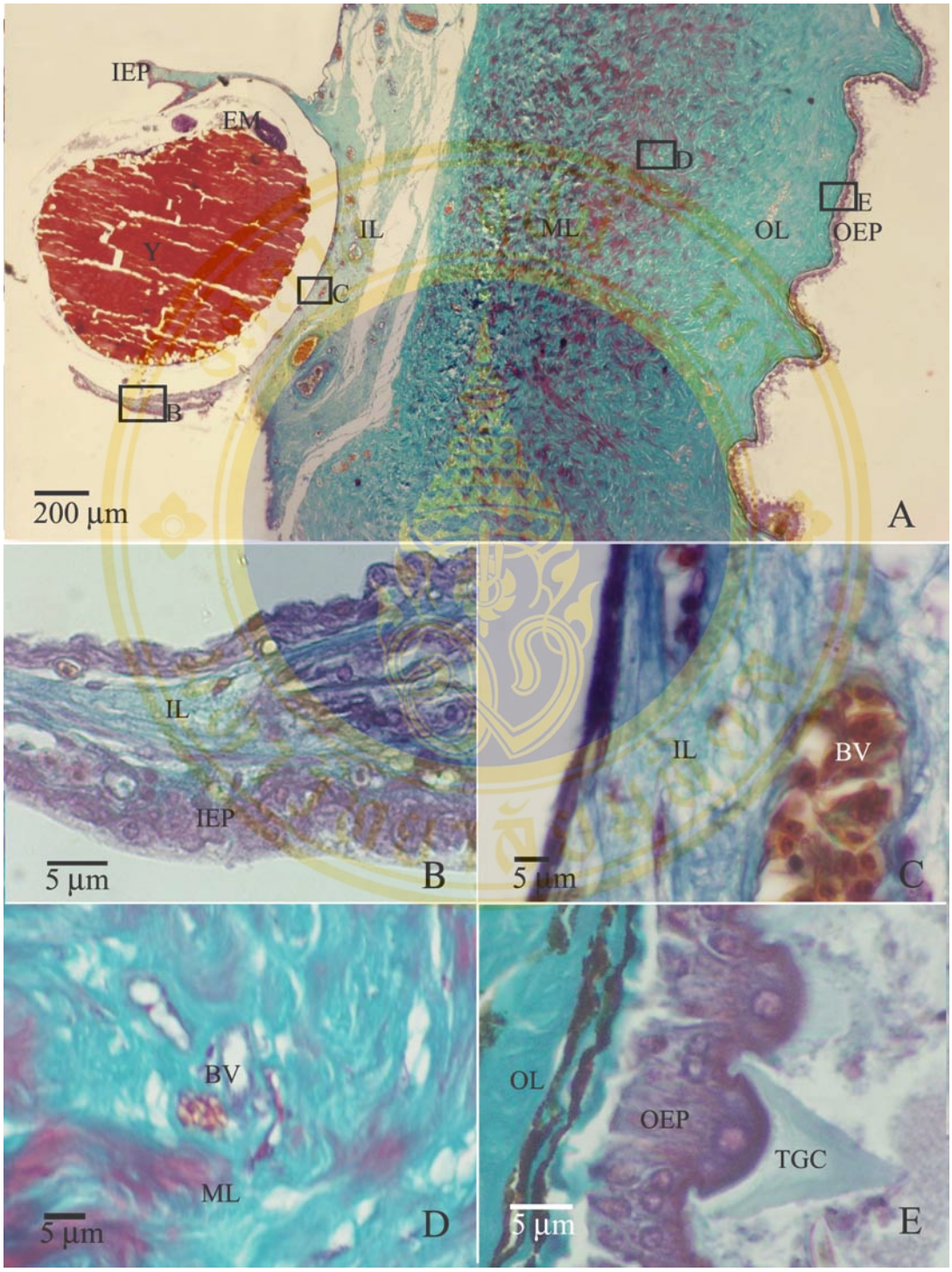
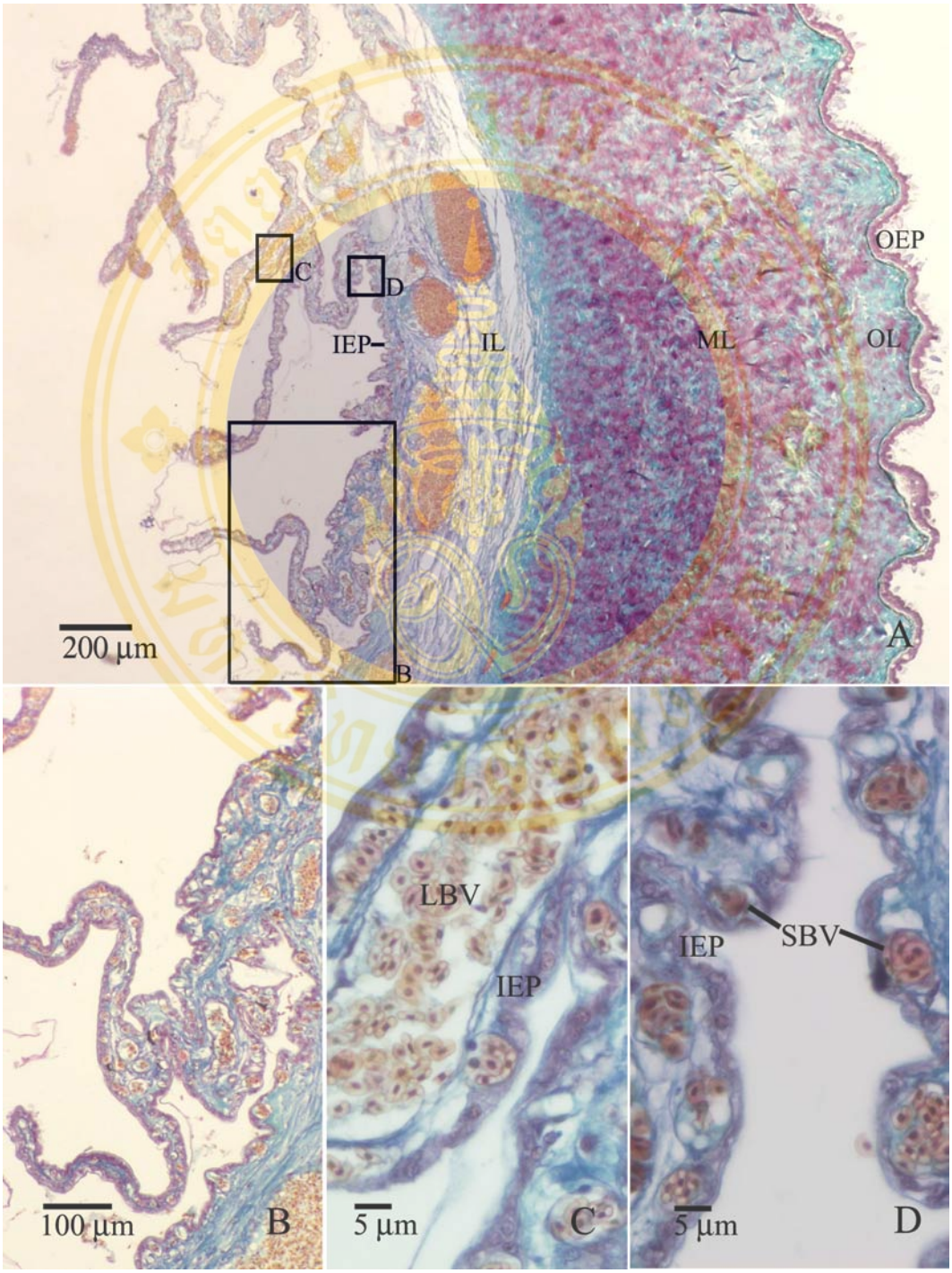


Figure 26. Embryo released stage in brooding male; A) Embryos has been released from the brood wall; B) Inner epithelium and tissue layer are flat and wrinkle; C) Large blood vessel (LBV) lies close to the inner epithelium (IEP); D) Small blood vessels (SBV) are located under the inner epithelium (IEP).



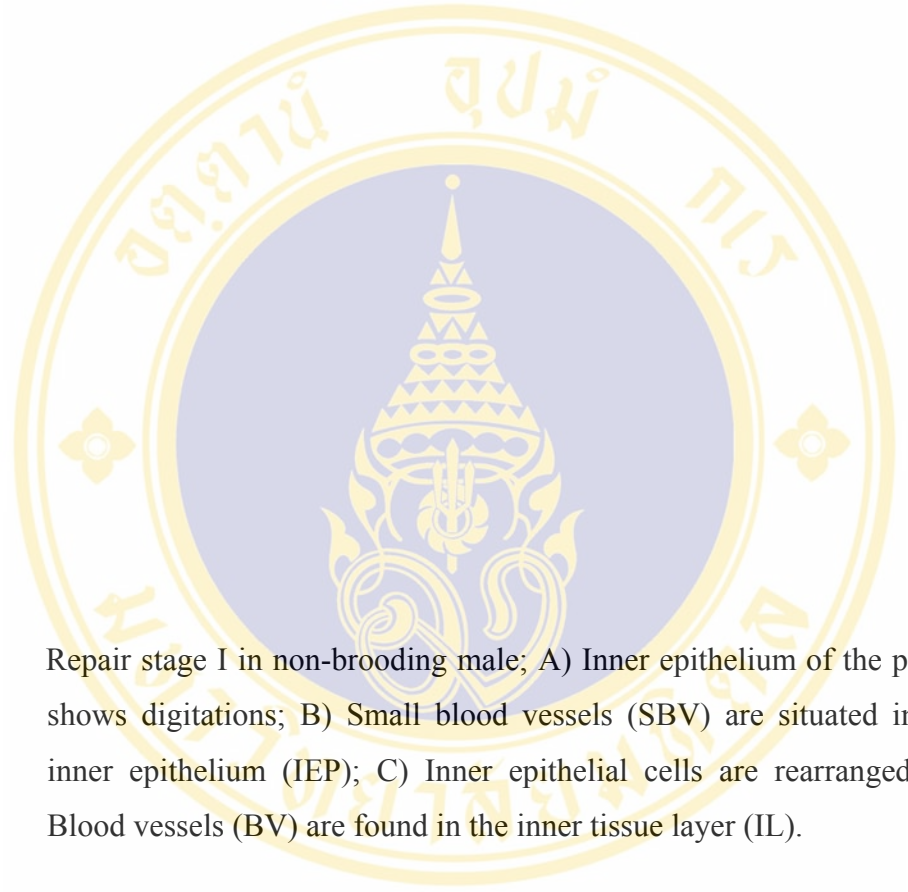


Figure 27. Repair stage I in non-brooding male; A) Inner epithelium of the pouch shows digitations; B) Small blood vessels (SBV) are situated in the inner epithelium (IEP); C) Inner epithelial cells are rearranged; D) Blood vessels (BV) are found in the inner tissue layer (IL).

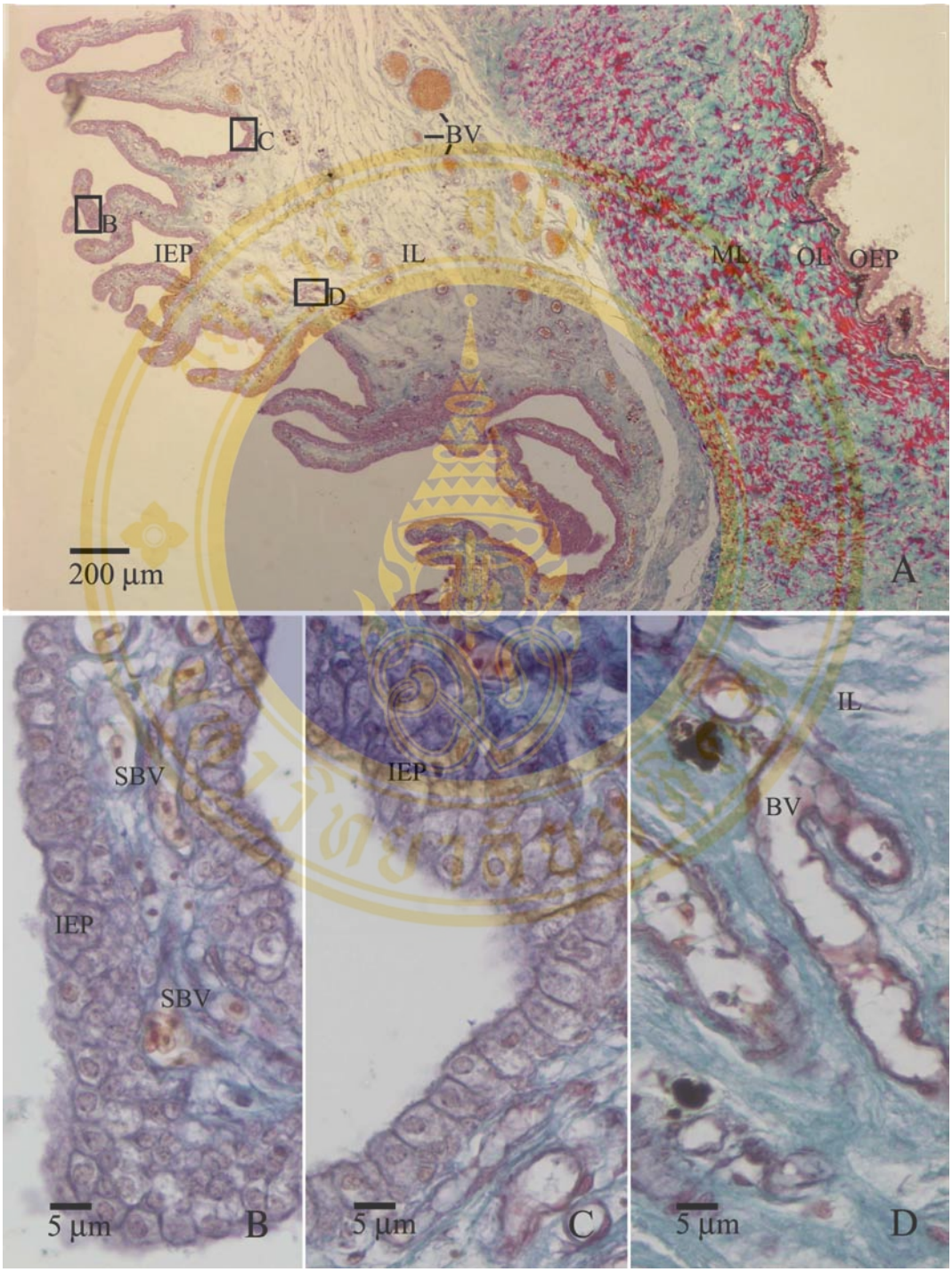
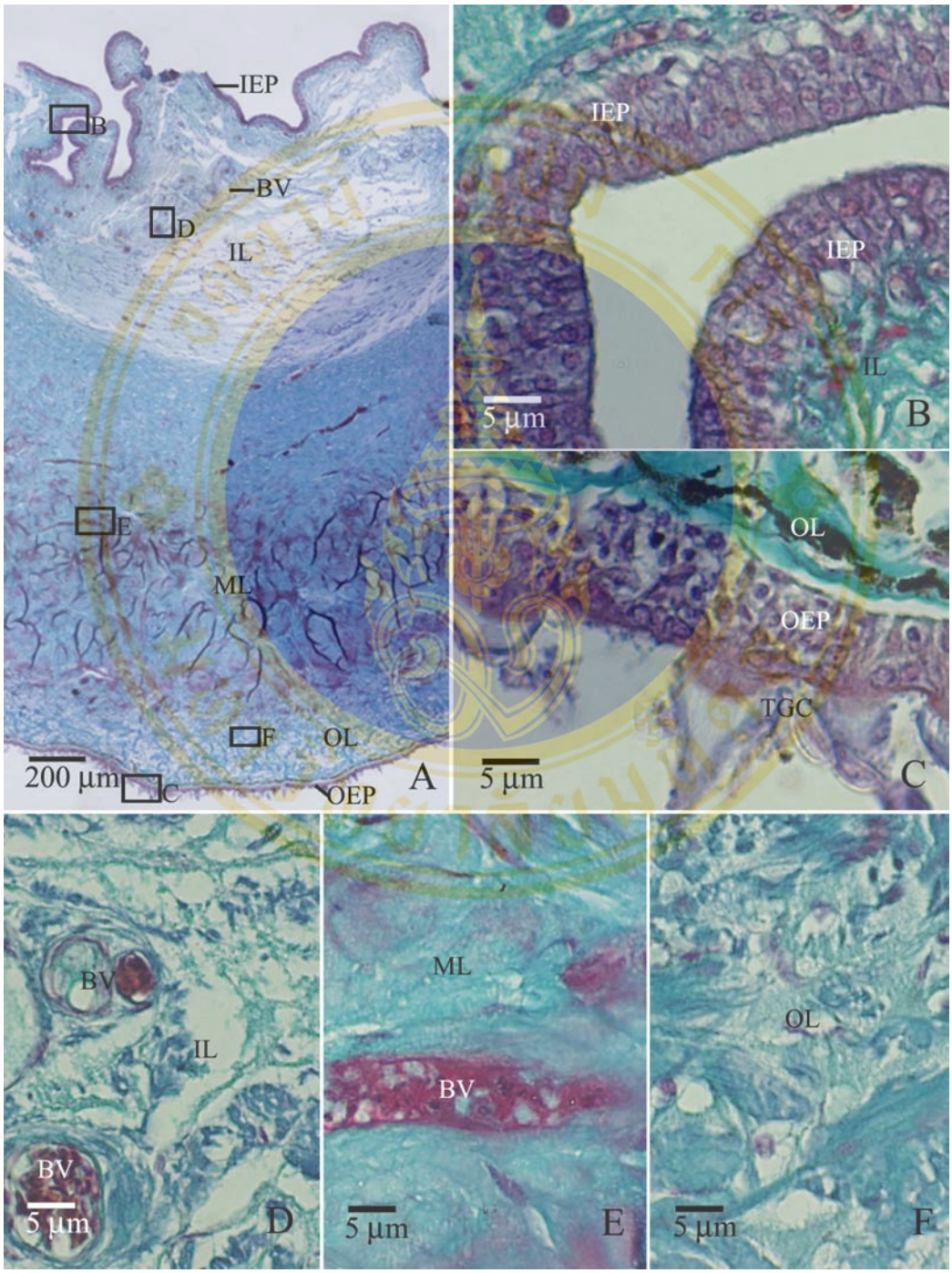


Figure 28. Repair stage II in non-brooding male; A) Inner epithelial layer and tissue layer are increase in thickness; B) Inner epithelium (IEP) that have been attached by embryo; C) Inner epidermal cells are rearranged and inner tissue layer (IL) is filled with loose connective tissue; D) Blood vessels (BV) in the inner tissue layer (IL); E) Blood vessels (BV) are found in the middle tissue layer (ML).



CHAPTER 6

DISCUSSION

Similar to many teleosts, testis of *H. kuda* is a pair of elongate semi-translucent sausage-like lobe located in the posterior of the body cavity (Carcupino, 1999; Koulish, 2002; Nostro, 2003). Most teleost testes consist of many germinal compartments (Grier et al., 1980), while *H. kuda* testis has only a single tubule. This was similar to the testis of pipefish (Carcupino et al., 1999). Therefore, the distribution of spermatogenic cells of the seahorse and pipefish is rather similar. The testis types can be defined into 2 types, restricted and unrestricted spermatogonia testis types, base on the distribution of spermatogonia. The type that spermatogonia are found only at the distal ends of the tubular testis is a restricted spermatogonia testis type. The testis of seahorse is an unrestricted spermatogonia type, since spermatogonia are distributed along the entire testis length.

During the reproductive season, various spermatogenic cells were observed in many teleost testes. In Florida gar, Japanese huchen, Eurasian perch and spotted halibut, their testes consist of spermatogonia, spermatocytes, spermatids and spermatozoa (Amer et al., 2001; Koya et al., 2003; Orlando et al., 2003; Sulistyo et al., 2000) while some teleost testes contain only some spermatogenic cell types. Spermatids and spermatozoa are found in testis of golden rabbit fish (Rahman et al., 2000), Only spermatozoa are found in the Pacific herring testis (Koya et al., 2002), spermatocytes, spermatids and spermatozoa are found in the black rockfish (Mori et al., 2003) and spermatogonia, primary spermatocytes, spermatids and spermatozoa are found in pipefish testis (Carcupino et al., 1999). Interestingly, all of them contain spermatozoa. In the present study, all stages of spermatogenic cells were found in the *H. kuda* testis except for spermatozoa. From previous studies, most teleosts had high percentage of spermatozoa during reproductive season (Amer et al., 2001; Koya et al., 2002; Koya et al., 2003; Orlando et al., 2003; Mori et al., 2003; Rahman et al., 2000; Sulistyo et al., 2000; Weltzien et al., 2002) while, in *H. kuda*, the percentage of

secondary spermatocytes was the highest both in brooding and non-brooding males. There are many explanations for such lack of spermatozoa. Firstly, unlike other teleosts, reproductive system of *H. kuda* is not compartmentalized which thus lacks sperm storage region. Spermatozoa may only be found in the testes during courtship. Secondly, the highest percentage of secondary spematocytes is probably indicates that the process of spermatogenesis is temporarily stopped at the secondary spermatocyte stage. These cells are stored in the testes until they are synchronously induced to form spermatids. Secondary spermatocytes are surrounded by fibrilar materials. The materials may provide essential materials for the cells to survive for a long time till spermatogenesis completes. Thirdly, Vincent (1995) suggested that the male seahorse can remate after releasing their offsprings during the breeding season. Therefore, the process of sperm production does not seem to take very long. Fourthly, seahorses do not move around much because of their reduced fins. So, they have a small home range and difficulty in finding a partner (Teixeira and Musiuck, 2001). This results in monogamy of mating system. Commonly, the mating pair of seahorses will stay together for all their lives (Kvarnemo et al., 2000). Thus, it may not be necessary for the males to store spermatozoa inside the testes during reproductive season. Lastly, seahorse has internal fertilization; unlike other teleosts, fertilization of seahorse occurs in the male. During copulation, the female transfers unfertilized eggs into the male pouch where fertilization takes place (Jones and Avise, 2001). The testes and the brood pouch are connected by a small pore. The spermatozoa can be directly transported into the brood pouch. Therefore, the complex structure of sperm storage compartment is not essential. These are possible reasons indicating that seahorse may produce sperm only when they are to mate.

In teleosts, sex hormones, i.e., estradiol, testosterone and progesterone have important roles in various aspects of testicular development. In some teleosts, estradiol is necessary for mitotic phase in spermatogenesis. Amer et al. (2001) suggested that estradiol in Japanese huchen promotes spermatogonial renewal. Low serum level of estradiol in both brooding and non-brooding males detected in the present study corresponds with the low numbers of early spermatogenic cells found in the testes. The other hormones, testosterone and progesterone, are essential for development of late spermatogenic cells. Testosterone functions in early

spermatogenesis and spermiogenesis in Eurasian perch (Sulistyo et al., 2000) while progesterone plays a role in sperm maturation in golden rabbit fish (Rahman et al., 2000). Progesterone promotes milt production, induces sperm motility in Pacific herring (Koya et al., 2002) and regulates spawning behavior in Japanese huchen (Amer et al., 2001). Thus, lacking of the significant level of both testosterone and progesterone in the present study corresponds with the lacking of late spermatogenic cells.

Limited information on the brood pouch is due to the fact that brood pouch is observed only in the Syngnathidae family and that the members of this family are not commercial fish (Vincent, 1992). Brood pouch morphology of *H. kuda i*s similar to that of *H. hippocampus*. Carcupino (2002) investigated brood pouch of brooding male in *H. hippocampus* and reported that it contained pear-shaped eggs and the embryos are in synchronous development. Belonging to the same family (Syngnathidae), male pipefish also has brood pouch, but its morphology differs from that of seahorse. The pouch is less complex and is located ventrally in the tail region. The pouch consists of two folds of skin covering the round eggs (Herald, 1959).

Carcupino (2002) studied the ultrastructure of brood pouch in *H. hippocampus*. The pouch epidermis is thin and folded. The basal epithelial layer lies closely to dermis and forms many interdigitations. The outer epithelium is composed of 2 different cell types: the classic filament-containing cells and the mitochondria-rich cells. The dermis appears much more vascularized containing many large capillaries. Previous reports on brood pouch did not include comparison of brood pouches in brooding and non-brooding males. The present study indicates variation of brood pouch morphology in various stages. Brood pouch composed of 5 different stages. Normal stage in non-brooding male shows thick inner epithelium and inner tissue layer. This stage is ready for embryo attachment. Two stages of brooding pouch can be differentiated, the stage at which embryos are attached and the stage at which embryos have been released. Upon the release of embryos, thus, 2 other stages of the non-brooding pouch could be identified, the repair stage I and stage II. The inner epithelium of repair stage I stage in non-brooding pouch shows digitations and the tissue layers contain blood vessels. This stage may be the stage following the release of embryos since several features of the inner epithelium and tissue layers of the pouch

are similar to the pouch of brooding male which embryos have been released. The repair stage II follows the repair stage I. Expansions of inner tissue layer and increasing in the size of inner epithelial cells are the characteristics of repair stage II. Characteristics of the inner epithelium and tissue layer, distribution of blood vessels are used for brood pouch staging. After the release of embryos, the blood vessels seem to be gradually reduced in size. The distribution of smooth muscles is also changed during brooding. The smooth muscles which are located originally in the middle tissue layer become dispersed into the middle and the outer tissue layers during the stage of embryo released. But gradually relocalized in the middle layer during the repair stage. The dynamics of the smooth muscles may involve in the mechanism of the embryo release.

CHAPTER 7

CONCLUSIONS

The present study compared germ cell development and brood pouch morphology between brooding and non-brooding male seahorses. The following conclusions can be made:

1. Testicular morphology, histology and proportion of spermatogenic cells between brooding and non-brooding males were similar.

2. The testes were paired, elongated and semitranslucent-like organs containing various spermatogenic cells: spermatogonia, 7 stages of primary spermatocytes (leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase and anaphase), secondary spermatocytes and spermatids. Secondary spermatocytes were the highest percentage followed by primary spermatocytes, spermatogonia and spermatids, respectively.

3. The sex hormone levels, estradiol, testosterone and progesterone, between brooding and non-brooding males were not different.

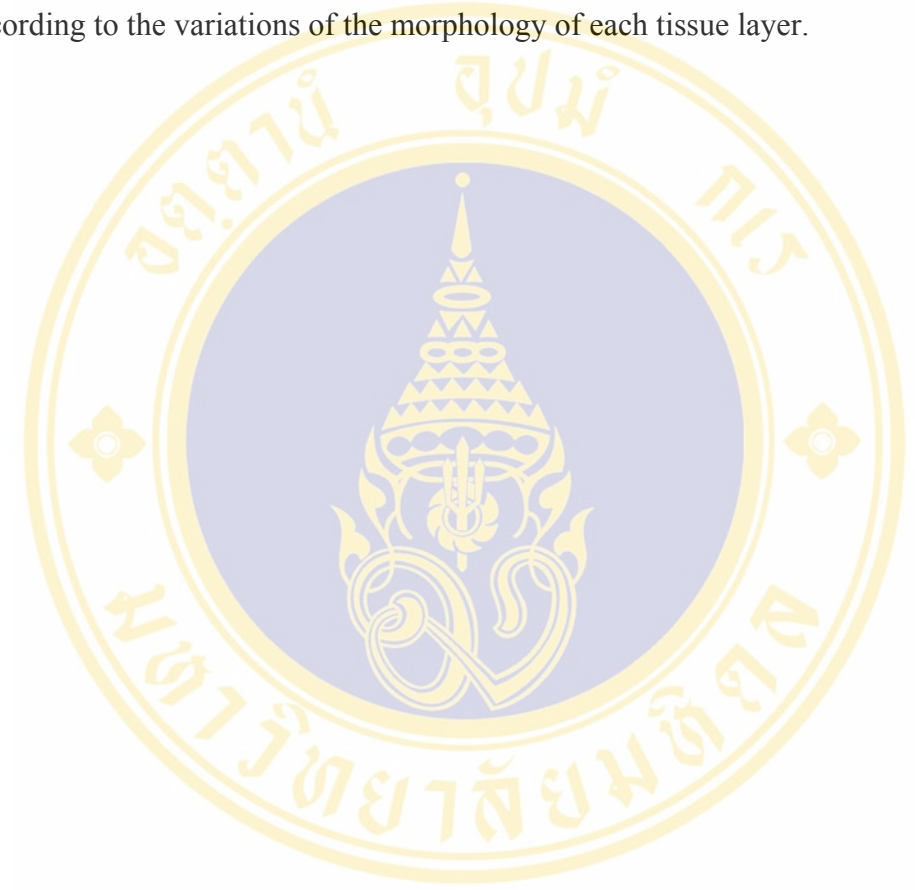
4. Brood pouch of brooding male was different from that of non-brooding male. The brooding pouch was enlarged due to the presence of embryos and was highly vascularized while that of the non-brooding male was flat and had fewer blood vessels.

5. Brood pouch wall was composed of 2 epithelial layers: inner (pseudostratified) and outer epithelia (stratified cuboidal). Between the 2 epithelial layers, there were 3 tissue layers: inner (loose connective tissues), middle (smooth muscles) and outer tissue layer (dense irregular connective tissues); the inner tissue layer contains various sizes of blood vessels.

6. The inner epithelium and inner tissue layer of non-brooding pouch were thick; relatively few blood vessels were found in the inner tissue layer and the smooth muscles were localized in the middle tissue layer.

7. The inner epithelium and tissue layers of the brooding pouch in the brooding male were thin; there were abundant blood vessels in the inner tissue layer and the smooth muscles were distributed in the middle and the outer tissue layers.

8. The brood pouch can be differentiated into 5 different stages: normal stage, embryo carrying stage, embryo released stage, repair stage I and repair stage II according to the variations of the morphology of each tissue layer.



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APPENDIX

APPENDIX A

Paraffin technique and staining solution

Solution for paraffin technique

1. Fixative Bouin's solution

Piric acid (saturated aqueous)	50	ml
40% formaldehyde	250	ml
Gracial acetic acid	50	ml

Staining solution

1. Harris's hematoxy solution

Hematoxylin crystals	5	g
Ethyl alcohol, 95%	50	ml
Potassium or ammonium alum	100	ml
Distilled water	950	ml
Mercuric acetic acid	2.5	g
Gracial acetic acid	1-2	ml

2. Alcoholic eosin solution

Eosin-Y	2	g
Distilled water	160	ml
Ethyl alcohol, 95%	640	ml

3. Masson Trichrome stain

3.1 Acid fusin

Acid fusin	1	g
Distilled water	100.0	ml
Glacial acetic acid	1.0	ml

3.2 Ponceau S

Ponceau S	1	g
Distilled water	100.0	ml
Glacial acetic acid	1.0	ml

3.3 Fast green

Fast green	2	g
Distilled water	100.0	ml
Glacial acetic acid	1.0	ml

3.4 Phosphotungstic acid

Phosphotungstic acid	1.5	g
Distilled water	100.0	ml
Glacial acetic acid	1.0	ml

4. Acidified water

Glacial acetic acid	0.2	ml
Distilled water	100	ml

APPENDIX B

Weight and length of the bodies or testes

Table B-1 Weight and length of the body and testis of the brooding males collected each month.

Month	Seahorse No.	Body length (cm)	Testis length (cm)	Body weight (g)	Testis weight (g)	Testis length/Body length
Dec-02	1	16.60	1.50	18.256	0.073	0.090
	2	16.00	1.50	16.518	0.060	0.094
	3	15.00	1.45	14.221	0.052	0.097
	4	14.50	1.40	14.212	0.058	0.097
	5	15.80	1.50	15.660	0.075	0.095
	6	14.50	1.40	13.720	0.075	0.097
	7	11.30	1.25	5.415	0.030	0.111
	8	11.00	0.85	9.952	0.050	0.077
	9	10.20	0.80	12.714	0.058	0.078
	Average	13.88	1.29	13.408	0.059	0.093
	SD	2.40	0.28	3.807	0.015	0.010
Jan-03	1	13.00	0.85	12.254	0.026	0.065
	2	16.00	1.45	16.708	0.08	0.091
	3	13.50	1.70	12.424	0.045	0.126
	4	12.70	0.56	11.008	0.017	0.044
	5	15.40	1.30	14.735	0.025	0.084
	Average	14.12	1.17	13.426	0.039	0.082
	SD	1.49	0.46	2.275	0.025	0.031
Feb-03	1	13.50	0.85	12.118	0.016	0.063
	2	12.60	1.10	9.300	0.024	0.087
	3	12.80	0.75	9.266	0.013	0.059
	4	16.20	1.40	12.529	0.03	0.086
	5	16.60	1.40	23.556	0.032	0.084
	6	14.70	1.00	15.714	0.024	0.068
	7	13.70	0.90	16.977	0.029	0.066
	8	12.00	0.80	7.592	0.017	0.067
	Average	14.01	1.03	13.382	0.023	0.073
	SD	1.68	7.31	5.228	0.007	0.012
Mar-03	1	15.50	1.10	22.821	0.031	0.071
	2	14.90	0.90	16.609	0.02	0.060
	3	13.60	0.80	14.953	0.015	0.059
	4	14.20	1.10	15.047	0.025	0.077
	5	13.00	1.10	12.573	0.01	0.085
	6	15.00	1.30	21.732	0.06	0.087
	7	13.30	0.90	13.705	0.019	0.068
	Average	14.21	1.03	16.777	0.023	0.072
	SD	0.95	0.17	3.969	0.017	0.011

Table B-2 Weight and length of the body and testis of the non-brooding males were collected each month.

Month	Seahorse No.	Body length (cm)	Testis length (cm)	Body weight (g)	Testis weight (g)	Testis length/Body length
Dec-02	1	17.00	1.50	17.980	0.079	0.088
	2	16.60	1.50	6.280	0.028	0.090
	3	13.50	1.35	8.647	0.050	0.100
	4	13.00	1.30	12.831	0.081	0.100
	5	14.00	1.40	10.694	0.074	0.100
	6	11.50	0.55	6.548	0.031	0.048
	7	9.50	0.65	3.684	0.020	0.068
	8	9.00	0.60	3.916	0.024	0.067
	9	9.20	0.65	3.987	0.022	0.071
	Average	12.59	1.06	8.285	0.045	0.081
	SD	3.03	0.43	4.827	0.026	0.019
Jan-03	1	11.00	1.20	7.880	0.016	0.109
	2	15.00	1.45	12.455	0.045	0.097
	3	14.50	1.40	12.397	0.043	0.097
	4	12.50	1.20	7.477	0.024	0.096
	5	16.60	1.46	15.326	0.054	0.088
	Average	13.92	1.34	11.107	0.036	0.097
	SD	2.19	0.13	3.349	0.016	0.008
Feb-03	1	12.80	0.90	9.822	0.020	0.070
	2	15.50	1.70	18.329	0.040	0.110
	3	11.00	0.50	5.481	0.011	0.045
	4	14.60	1.10	11.309	0.030	0.075
	5	14.00	1.20	11.623	0.032	0.086
	6	11.50	0.90	6.868	0.019	0.078
	Average	13.23	1.05	10.572	0.025	0.077
	SD	1.78	0.40	4.517	0.011	0.021
Mar-03	1	16.2	0.9	18.881	0.024	0.056
	2	15.0	1.2	8.050	0.053	0.080
	3	13.0	0.8	6.530	0.010	0.062
	4	14.7	0.9	11.484	0.021	0.061
	5	11.4	0.5	5.820	0.010	0.044
	6	12.9	0.7	7.405	0.014	0.054
	Average	13.9	0.8	9.695	0.022	0.059
	SD	1.74	0.23	4.911	0.016	0.012

APPENDIX C

Cell and nucleus size of spermatogenic cell types

Table C-1 Cell and nucleus size of spermatogenic cell types of the brooding and non-brooding males.

Seahorse No.	Spermatogonia (μm)		Primary spermatocyte (μm)	Secondary spermatocyte (μm)		Spermatid (μm)	
	cell	nucleus	cell	cell	nucleus	cell	nucleus
1	7.50	4.38	13.13	6.25	3.75	3.13	1.88
2	7.19	4.69	11.25	6.25	3.75	3.75	1.88
3	7.50	5.00	10.63	6.25	3.75	2.81	1.56
4	8.13	5.00	9.69	6.25	4.38	3.13	1.56
5	7.50	5.00	11.88	6.88	4.06	3.13	1.88
6	8.75	5.31	11.88	6.25	3.75	3.75	1.56
7	8.75	5.00	10.00	6.88	4.06	3.13	1.56
8	8.13	5.63	11.88	5.63	4.06	3.75	1.56
9	7.50	5.31	12.19	6.88	4.06	2.50	1.56
10	7.81	5.31	13.75	6.88	4.06	2.50	1.56
11	8.13	5.31	10.63	8.75	4.06	3.75	2.19
12	7.81	5.31	10.63	6.88	4.06	3.44	1.56
13	10.31	5.94	10.63	6.25	4.38	4.06	1.56
14	8.75	5.31	12.50	6.25	4.38	3.44	1.88
15	7.50	5.31	11.25	6.25	3.44	3.75	2.19
16	8.75	4.69	11.25	6.88	3.44	3.44	2.19
17	8.44	5.63	11.25	6.88	4.06	3.44	2.19
18	9.38	5.94	12.50	7.50	4.38	2.81	1.88
19	7.50	4.38	13.13	6.88	4.06	3.75	2.19
20	10.63	5.63	12.50	5.94	3.44	3.44	1.88
21	8.75	5.31	9.06	7.19	3.75	4.06	2.19
22	7.50	5.00	10.63	7.50	4.06	3.44	2.50
23	8.44	5.00	11.25	7.50	4.69	4.06	2.50
24	7.50	5.31	9.38	7.50	4.06	3.44	2.19
25	8.44	5.00	9.38	7.19	3.75	3.13	1.88
26	7.50	5.63	11.25	7.19	4.06	3.75	2.19
27	8.44	5.63	11.56	6.88	4.06	3.44	2.19
28	6.88	5.00	11.88	7.19	4.06	4.06	1.88
29	7.19	5.31	13.75	6.56	3.75	3.44	2.50
30	6.88	5.63	12.50	7.50	4.69	3.75	2.19
31	8.13	5.63	10.94	6.88	4.06	4.06	2.19
32	6.88	5.00	11.25	6.88	4.06	3.75	2.19
33	7.81	5.63	10.00	8.13	4.06	4.38	1.88
34	8.75	5.94	9.69	6.88	4.38	4.06	2.50
35	8.44	5.00	11.56	8.13	4.06	4.06	2.19

Table C-1 Cell and nucleus size of spermatogenic cell types of the brooding and non-brooding males (CONT.).

Seahorse No.	Spermatogonia (μm)		Primary spermatocyte (μm)	Secondary spermatocyte (μm)		Spermatid (μm)	
	cell	nucleus	cell	cell	nucleus	cell	nucleus
37	7.19	5.00	10.63	8.75	5.63	3.44	2.19
38	7.19	5.00	13.75	7.19	4.06	3.44	1.88
39	7.50	5.00	13.13	7.50	3.75	3.44	2.19
40	7.50	5.00	11.25	7.50	4.06	3.75	2.19
41	7.50	5.31	10.94	6.25	4.06	3.44	2.19
42	8.75	5.94	10.94	7.81	4.06	4.06	1.88
43	8.13	5.31	12.50	6.88	4.38	4.06	1.88
44	8.13	5.63	11.88	6.56	3.75	4.06	2.19
45	8.75	4.69	10.63	7.50	4.06	3.44	1.88
46	8.13	5.63	10.63	6.56	3.75	3.44	2.50
47	8.13	5.63	10.63	7.81	4.69	3.75	2.19
48	7.81	5.31	11.25	7.81	4.69	3.75	2.19
49	8.44	5.00	12.50	7.50	4.06	4.06	2.50
50	8.75	5.00	10.94	5.94	3.75	3.44	2.19
Average	8.05	5.26	11.35	6.99	4.07	3.59	2.03
SD	0.79	0.38	1.18	0.69	0.38	0.42	0.29

APPENDIX D

Number of spermatogenic cell types

Table D-1 Number of spermatogenic cell types of the brooding males each month.

Month	Seahorse No.	Spermatogonia (cells)	Primary spermatocyte (cells)	Secondary spermatocyte (cells)	Spermatid (cells)
Dec-02	1	25	50	25	0
	2	0	0	0	0
	3	0	0	0	0
	4	30	20	48	2
	5	33	33	33	1
	6	28	40	30	2
	7	30	24	45	1
	Average	20.9	23.9	25.9	0.9
	SD	14.45	19.07	19.42	0.90
Jan-03	1	20	27	50	3
	2	20	20	58	2
	3	17	20	60	3
	4	33	33	33	1
	5	18	40	40	2
	Average	21.6	28	48.2	2.2
	SD	6.50	8.63	11.58	0.84
Feb-03	1	15	35	45	5
	2	12	35	50	3
	3	10	35	50	5
	4	25	25	46	4
	5	10	35	50	5
	6	15	30	50	5
	Average	14.5	32.5	48.5	4.5
	SD	5.61	4.18	2.35	0.84
Mar-03	1	24	24	48	4
	2	16	35	45	4
	3	16	35	47	2
	4	16	38	40	6
	5	13	40	45	2
	6	15	40	40	5
	7	17	41	40	2
	Average	16.7	36.1	43.6	3.6
	SD	3.45	5.87	3.51	1.62

Table D-2 Number of spermatogenic cell types of the non-brooding males each month.

Month	Seahorse No.	Spermatogonia (cells)	Primary spermatocyte (cells)	Secondary spermatocyte (cells)	Spermatid (cells)
Dec-02	1	10	45	35	10
	2	15	50	35	0
	3	30	35	35	0
	4	13	40	40	7
	Average	17	42.5	36.25	4.25
	SD	8.91	6.45	2.50	5.06
Jan-03	1	20	30	40	10
	2	17	30	40	13
	3	17	30	40	13
	4	15	30	40	15
	5	11	30	50	9
	Average	16	30	42	12
	SD	3.32	0.00	4.47	2.45
Feb-03	1	21	30	42	7
	2	13	30	45	12
	3	15	25	45	15
	4	14	26	50	10
	5	13	30	50	7
	Average	15.2	28.2	46.4	10.2
	SD	3.35	2.49	3.51	3.42
Mar-03	1	13	40	40	7
	2	20	30	50	0
	3	15	30	45	10
	Average	16	33.3	45	5.67
	SD	3.61	5.77	5.00	5.13

APPENDIX E

Statistical analysis

Table E-1 Average weight and length of the body and testis of the brooding and non-brooding males.

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Measurement	T	df	P	Brooding male	Non-brooding male	Total
Body length	1.44	53	0.1559 (NS)	14.04±1.70 N = 29	13.28±2.30 N = 26	13.68±2.02 N = 55
Body weight	3.93	53	0.0002*	14.21±4. 15 N=29	9.68±4.41 N=26	12.07±4.81 N=55
Testis length	-0.70	35	0.4909 (NS)	1.13±0.30 N = 29	1.06±0.36 N = 26	1.10±0.33 N = 55
Testis weight	0.68	53	0.4990 (NS)	0.038±0.02 N = 29	0.034±0.02 N = 26	0.036±0.02 N = 55

NS = not significantly different ($P<0.05$) * = significantly different

Table E-2 Percentage of spermatogenic cell types of the brooding and non-brooding males.

Spermatogenic cell type	T	df	P	Brooding male	Non-brooding male	Total
Spermatogonia	1.10	38.4	0.28 (NS)	18.32±8.79 N=25	16.00±4.74 N=17	17.38±7.44 N=42
Primary spermatocytes	-0.97	39.1	0.34 (NS)	30.20±11.83 N=25	33.00±6.79 N=17	31.33±11.09 N=42
Secondary spermatocytes	-0.55	32.3	0.59 (NS)	40.72±14.64 N=25	42.47±5.30 N=17	41.43±11.72 N=42
Spermatids	-4.72	19	0.0001	2.76±1.79 N=25	8.53±4.82 N=17	5.09±4.38 N=42

* = significantly different NS = not significantly different

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