Reproductive Biology of Short-neck Clam *Marcia recens* (Holten, 1802) at Tambon Koh Sarai, Satun Province

Wirot Kongasa

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Master of Science in Aquatic Science

Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ ชีววิทยาการสืบพันธุ์ของหอยกะหยำ *Marcia recens* (Holten, 1802) บริเวณตำบลเกาะสาหร่าย จังหวัดสตูล

ผู้เขียน นายวิโรจน์ คงอาษา

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ปีการศึกษา 2561

บทคัดย่อ

การศึกษาวงจรการสืบพันธุ์ของหอยกะหยำ (short-neck clam) *Marcia recens* (Holten, 1802) ดำเนินการใน 2 พื้นที่ (เปลายัน และช่องหลาด) ของตำบลเกาะสาหร่าย จังหวัดสตูล ชายฝั่งทะเลอันดามัน ภาคใต้ของประเทศไทย โดยรวบรวมตัวอย่างหอยกะหยำทั้งหมด 1,167 ตัว (ความยาวเปลือก 12.25 - 48.50 มิลลิเมตร) ตั้งแต่เดือนพฤศจิกายน 2552 ถึงเดือนกุมภาพันธ์ 2554 ใช้เทคนิคด้านคุณภาพ (การวิเคราะห์ทางเนื้อเยื่อวิทยา) ตลอดจนเทคนิคเชิงปริมาณ (การวิเคราะห์ดรรชนีความสมบูรณ์ และดรรชนีความสมบูรณ์เพศ) ในการวิเคราะห์หาระยะพัฒนาการของระบบสืบพันธุ์ในแต่ละเดือน วงจรการสืบพันธุ์ และขนาดแรกเริ่มสืบพันธุ์ของหอยกะหยำ การวิเคราะห์ตัวอย่างเพื่อหาค่าความสัมพันธ์ระหว่างวงจรการสืบพันธุ์กับปัจจัยทางสิ่งแวดล้อม จากการศึกษาพบว่าหอยกะหยำเป็นหอยแบบแยกเพศ (dioecious) ระยะพัฒนาการระบบสืบพันธุ์ของหอยกะหยำเพศผู้และเพศเมียแบ่งออกได้ 6 ระยะ ได้แก่ ระยะที่ 0 คือระยะพัก ระยะที่ 1 คือระยะเริ่มต้นพัฒนาการ ระยะที่ 2 คือระยะปลายพัฒนาการ ระยะที่ 3 คือระยะสมบูรณ์เพศ ระยะที่ 4 คือระยะปล่อยเซลล์สืบพันธุ์ และระยะที่ 5 คือระยะปล่อยเซลล์สืบพันธุ์หมดแล้ว การเปลี่ยนแปลงค่าดรรชนีความสมบูรณ์เพศในแต่ละเดือนของหอยกะหยำทั้งสองพื้นที่ศึกษาแสดงให้เห็นว่าพัฒนาการระบบสืบพันธุ์ของหอยกะหยำทั้งสองเพศเกิดขึ้นตลอดทั้งปี ในขณะที่ค่าดรรชนีความสมบูรณ์ของหอยกะหยำทั้งสองพื้นที่ศึกษาไม่สอดคล้องกับพัฒนาการระบบสืบพันธุ์ของหอยกะหยำ ดัชนีความสมบูรณ์เพศของหอยกะหยำไม่มีความสัมพันธ์กับดัชนีความอุดมสมบูรณ์ ฤดูวางไข่สืบพันธุ์เกิดขึ้นตลอดทั้งปีโดยพบหนาแน่นระหว่างเดือนพฤศจิกายน - กุมภาพันธ์ อัตราส่วนเพศ (เพศผู้ : เพศเมีย) ของประชากรหอยกะหยำเท่ากับ 1 : 1.07 (χ^2 = 1.429, d.f. = 1, P <0.05) นอกจากนั้นยังพบหอยกะหยำที่มีสองเพศในตัวเดียวกันเพียงแค่ 3 ตัวในพื้นที่เปลายัน ขนาดแรกเริ่มสืบพันธุ์ของหอยกะหยำเพศผู้และเพศเมีย มีความยาวเปลือก 19.51 และ 20.09 มิลลิเมตร ตามลำดับ ปัจจัยสิ่งแวดล้อมบริเวณแหล่งที่อยู่อาศัยของหอยกะหยำที่ศึกษาในแต่ละเดือน ประกอบด้วยอุณหภูมิ ความเค็ม ความเป็นกรด-ด่าง ปริมาณออกซิเจนที่ละลายในน้ำ คลอโรฟิลล์ เอ อินทรียวัตถุในดิน และโครงสร้างของดิน ความสัมพันธ์ระหว่างปัจจัยสิ่งแวดล้อมกับพัฒนาการระบบสืบพันธุ์ของหอย

กะหยำในทั้งสองพื้นที่ศึกษา พบว่าความเค็มมีความสัมพันธ์เชิงลบอย่างมีนัยสำคัญกับพัฒนาการระบบสืบพันธุ์ของหอยกะหยำเพศเมียระยะปล่อยเซลล์สืบพันธุ์ในพื้นที่ศึกษาเปลายัน (Pearson product-moment correlation, r = -0.543, n = 16, *P* = 0.03) หอยกะหยำจากตำบลเกาะสาหร่าย จังหวัดสตูล ภาคใต้ของประเทศไทยมีการเกิดขึ้นใหม่อย่างต่อเนื่องโดยมีช่วงชุกชุมอยู่ 2 ช่วง ซึ่งแสดงให้เห็นถึงรูปแบบของฤดูกาลสืบพันธุ์วางไข่

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Academic Year	2018

ABSTRACT

A study of the reproductive cycle of the Short-neck clam *Marcia recens* (Holten, 1802) was conducted at two different areas (Plao Yan and Chong Lad) of Koh Sarai sub-district, Satun Province on the Andaman sea coast of southern Thailand. A total of 1,177 samples (of between 12.25 - 48.50 mm shell length) were collected from November 2009 until February 2011. Qualitative techniques (histology analysis) as well as quantitative techniques (analysis of condition index and gonadal index) were used to investigate the monthly gonadal development stages, the reproductive cycle, and the size at first maturity of *M. recens.* The samples were analyzed to determine the relationship between the reproductive cycle and environmental factors. It was observed that *M. recens* is a dioecious clam. Six different gonad development stages in both males and females were identified: (0) resting stage, (1) early development stage, (2) late development stage, (3) ripe stage, (4) partially spent stage, and (5) spent stage. Monthly changes in the gonadal index in both sites indicated that gametogenesis occurred throughout the year, while the condition index in both sites did not explain gametogenesis. The gonad index of *M. recens* did not show any correlation to their condition index. The spawning season was identified as lasting all the year around, with a peak during November - February. The sex ratio (male: female) of the clam population was 1:1.07 (χ^2 = 1.429, d.f. = 1, P <0.05). Only three hermaphrodites were found at the Plao Yan site. Males attained their first sexual maturity at a shell length of 19.51 mm, while female maturity occurred at 20.09 mm. Environmental factors measured monthly in the habitat of *M. recens* included temperature, salinity, pH, dissolved oxygen, chlorophyll *a*, organic matter, and soil texture. The correlations between these factors and *M. recens* gametogenesis were calculated for both sampling sites. Only salinity was significantly negative correlated with the partially spent stage of females at Plao Yan (Pearson product-moment correlation, r = -0.543,

n = 16, P = 0.03). *Macia recens* from Tambon Koh Sarai, Satun Province, southern Thailand, displayed continuous recruitment with two peaks, which suggested a seasonal pattern of reproductive activities.

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I would like to sincerely acknowledge the efforts of many people who contributed to this thesis. Without them, the work would never has been completed.

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I am also indebted to Miss Janejit Kongkumnerd, Director, and the staff from the Aquatic Animal Health Research Institute, Songkhla, who supported and supplied facilities for my thesis. I am very grateful to many person who assisted me in collecting data in the field, particularly to Mr. Roh-Sone Nooprim and Mr. Whapee Suwalam, fishermen from Tanyongkling village, Koh Sarai subdistrict, Muang District, Satun Province, Thailand.

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Wirot Kongasa

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CHAPTER 1

Introduction

1.1 Importance and background

The short-neck clam *Marcia recens* (Holten, 1802) or locally called Hoi Ka-yam is a commercial important clam in Southern Thailand. They are targeted for local consumption and earned more income for coastal communities. They are commonly found along the coastal area of the Andaman sea of Thailand (Nateewathana, 1995) especially in the coastal area of Satun Province. Small scale fishermen in Satun Province collect this bivalve for family consumption and also sell it to local middlemen because of its better taste than other species found along the coast of Satun Province, such as *Meretrix* sp. Moreover, they also found in Pattani Bay, southern Gulf of Thailand (Swennen et al., 2001). The small scale fishermen in Pattani Bay collect these bivalves also for family consumption and sell in the local market of Pattani Province. This bivalve is medium size clam inhabits in shallow coastal water, so make it easier to collect by fishermen. In case of Satun Province, This bivalve is abundant around islands in Tambon Koh Sarai; compose of many islands approximately 69 islands in the Andaman Sea. The coastal area of this tambon is richness of many marine organisms because the variations of currents and physical processes associated with an island mass effect could influence biological processes as well as the settlement of some invertebrate larvae and productivity in the vicinity of, and around islands (Sander, 1971 cited by Khokiattiwong, 1991). Khokiattiwong (1991) believed that this might be one of the reasons why fishermen can collect snails or bivalves abundantly around some islands and also sea water condition is not contaminate with pollutants from the mainland. Local fishermen in this tambon collect this clam for many generations. They used coconut shell dig the soil during the low tide and pick the clams up by hand and keep in the container. Mr. Sa-pee Suwhalum (personal contact in October, 2009), local shell collector explained that in twenty years ago, he could collected the short-neck clam about 100 kg per day and there were plenty of them in Tambon Koh Sarai and he sold to local middleman with a price of 3 Baht per kilogram. Later, more fishermen collected this clam not only local fishermen but also fishermen from nearby Tambons. The fishermen also developed the gear to harvest these clams by using a hand dredge that made of stainless steel and they can collect more clams. At present, small scale fishermen in Satun Province have reported that the quantity of the short-neck clam has declined continually due to the high demand, especially from the export market to Malaysia (Mr.Sa-ad Lheepayang; personal contact in October, 2009) and the price of the short-neck clam that the fishermen can earn from local middlemen is 30 - 40 Baht per kilogram. For these reasons, short-neck clam has potentially economically important for coastal communities in Andaman sea of Thailand, if there is a proper management on exploitation for these bivalves.

Knowledge on the ecology and reproductive biology of bivalves is essential for the development of management schemes (e.g. spawning stock and/or larval settlement protection), and is important for establishment of successful hatchery based production. Understanding the gametogenesis and spawning season can also provide a suitable reproductive time for artificial spawning induction in aquaculture. A standpoint of management, this information is necessary to obtain healthy larvae and accordingly for promoting successful rehabilitation actions based on production from aquaculture. The understanding on the reproductive cycle of bivalves is also relevant for regulating dredge fishing, with implications for effective management (Joaquim et al., 2008). There are some studies concerns reproductive biology of bivalves that can be utilized for management strategies such as Peerakeitkachorn (1995) suggested that fishermen should not collect *Donax scortum* in the coastal area of Trang Province between February and July in each year because of their spawning season. Chatananthawej (1993) reported that the size at first maturity of short-neck clam *Paphia undulata* in Trat province was 3.2 cm. This led to dredge fishery management where the suitable distance between the slit of the dredge must not be less than 1.2 cm. Because of this, the small size short-neck clam can escape and have a chance to spawn and, at the same time, large short-neck clams are more valued than small size varieties.

The main objectives of the study on the reproductive biology of short-neck clams *Marcia recens* are to answer the question “How temporal and spatial differences affect reproductive characteristics of short-neck clams at Tambon Koh Sarai?” Concurrently, relevant environmental factors are also recorded.

This information can then be used for the management of short-neck clams in their natural habitats, and perhaps for aquaculture in the future.

1.2 Review of literatures

1.2.1 Shell morphology of *Marcia recens* (Holten, 1802)

Shell morphology of *M. recens* or locally called "Hoi Ka-yam" (Fig. 1) found in Tambon Koh Sarai, Mueang district, Satun Province is equivalve. Large ligament is located externally behind the umbo and in front of the umbo is the lunule that is easy to recognize. The umbo usually points to the anterior and commonly cream colored. The outsides of the valves are composed of concentric sculpture, fading out only near the posterior half of the ventral margin. The color varies between mostly dark brown and light brown, with dark brownish ray and blotches. The insides of the valves are composed of anterior and posterior adductor scars which are similar in size. The pallial line is easily to recognize. The pallial sinus is a deep ovate shape at the posterior and point out in horizontal. For small size valves, there is a pinkish colour inside the valves in some specimens. The lateral tooth is absent in both valves. There are three cardinal teeth in each valve radiating from the umbo. The median cardinal tooth of the left valve is rather narrow and slightly bifid, not appearing like two teeth; the posterior cardinal tooth of the right valve is also bifid. The internal margin is smooth.

There have been explanations the taxonomic status of this clam by Moore (1969), who distinguished *Marcia* from *Katelysia* from the characteristics of the outside of the shells, *Marcia*'s is smooth and *Katelysia*'s have concentric sculpture and irregular anteriorly and radial sculpture is weak. In both cases the outside of the shells have concentric sculpture but in *Marcia*'s radial sculpture is absent. According to the pallial sinus, *Marcia*'s is oval and horizontal while *Katelysia*'s is medium-sized to short.

In Thai waters, there are some studies identified the synonym of *Marcia recens* such as Nateewathana (1995) placed a morphologically of this clam from the Andaman sea coast in the genus *Marcia* (*Hemitapes*). The identification based on the morphology is: shell is ovate, thick, inflated; internal margins are smooth; irregular concentric sculpture; the outsides of the valve are brown with dark brownish rays and blotches and the insides of the valves are white. However, there was no mentioning on the cardinal teeth.

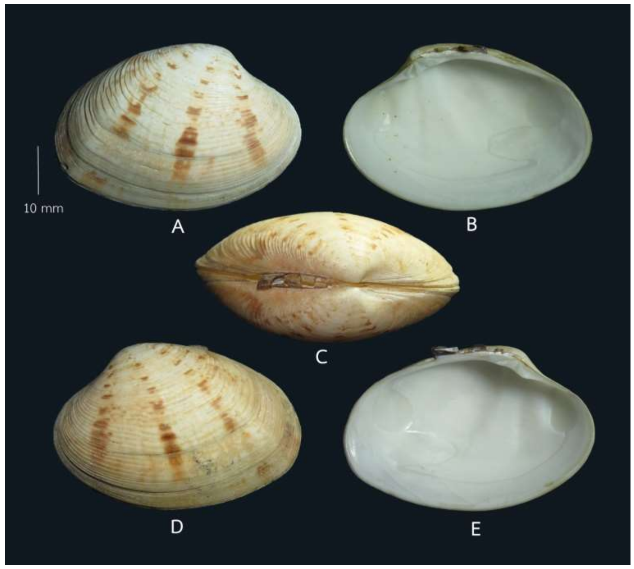

Figure 1 *Marcia recens* (Holten, 1802) **A** right valve dorsal **B** right valve ventral **C** dorsal margin **D** left valve dorsal **E** left valve ventral, Tambon Koh Sarai, Satun Province.

Later, Swennen et al. (2001) also identified this clam found in Pattani Bay, southern part of the Gulf of Thailand, as *Marcia marmorata*, where the shell is medium size, ovate to cordate in outline. The outside of the valves are composed of concentric sculpture or smooth. Lunule is large and not sunk, cardinal teeth are radiated and lack of a lateral tooth. The pallial sinus has an ovate shape and is relatively deep. The internal margin is smooth. Recently, Arathi et al. (2018) identified this clam found in Indian waters as *Marcia recens*, where its shell is robust, moderately thick, moderately inflated. Outline of the shell is elongate subovate and inequilateral, beaks are in front of midline. Lunule is flattened and not well defined. Escutcheon is weakly defined. Shell surface slightly glossy; sculpture commarginal, of weak lines and growth stops, some with more defined ridges especially over anterior area. Muscle scars are weakly heteromyarian, posterior larger. Pallial sinus is horizontally aligned, broadly rounded extending to one third of the shell length. External colouration highly variable and variously patterned, cream, red, white or brown and patterned with 3–4 black radiating rays, or darker trigonal blotches over a light ground or with anastomosing narrow radial rays. Internal colouration of shell is white, some with pinkish umbonal cavity.

1.2.2 Taxonomy details of *Marcia recens* (Holten, 1802)

The taxonomic account of short-neck clam down to the genus level is follow the explanation of Moore (1969) and species identification details are follow Arathi et al. (2018).

Phylum Mollusca

Class Bivalvia

Subclass Heterodonta

Order Veneroida

Superfamily Veneracea

Family Veneridae

Subfamily Tapetinae

Genus *Marcia*

Species *Marcia recens*

Scientific name: *Marcia recens* (Holten, 1802)

Common name: short-neck clam

Local name: Hoi Ka-yam

1.2.3 Biological characteristics of *Marcia recens* (Holten, 1802)

There are approximately 31,000 species of bivalves reported around the world. They have a shape more constant than gastropods; composed of two valves of shell, attached to each other by hinge teeth and filament structure called ligament. The growth of bivalves is expanding of the mantle and shell. All body parts include internal organs and the feet are covered by mantle and lack of the head. Most bivalves inhabit sea water, however, there are some species found in brackish and freshwater. They are never found on land because the general basic characteristic of bivalves is filter feeder. The bivalves can adapt to live abundantly in the sea because they can feed on phytoplankton mostly found in the sea (Uppatham et al., 1995). *M. recens* is same as other bivalve species in having hard shells to cover the body for protection from danger and fluctuations of environmental conditions. In general, the body of clams is divided into four parts: head, foot, visceral mass, and mantle. The mantle is the only organ found in animals in the Phylum Mollusca, and its function is to build the shell to cover the visceral mass. The inside of the shell consists of the body that is covered by the mantle, lack of head and uses gills to filter food particles from water intake. It has a strong foot muscle for movement and burrowing. It has short siphons and lives buried just beneath the surface of muddy-sand flats (http://mangrove.nus.edu.sg, 2009).

In Satun province, *M. recens* can be found throughout the year, but the quantity may vary. The local fishermen who harvested this clam explained that there are more clams in dry season than rainy season, but their size is smaller. The fishing period for these clams occurs during the lowest tide of spring tide of a full moon and new moon, since the water depth is less, and the duration of the low tide is longer. The main habitats of *M. recens* are coastal areas surround islands scattered in Koh Sarai archipelago and the river mouth of Mambang canal in Mueang district and the river mouth of Langu canal and the river mouth of the main canal in Ban Bo-chet-luk of Langu district (Mr. Wapee Suwhalum, personal contact in October, 2009).

1.2.4 Size and distribution of *Marcia recens* (Holten, 1802)

The maximum shell length of *M. recens* was recorded at 5.0 cm, more commonly as 4.00 cm, with was similar to the shell length of this clam found in Ashtamudi Lake and Tuticorin, Indian water that ranged from 1.2 - 5.1 cm (Arathi et al., 2018). It is a shallow burrower of sand and muddy-sand bottoms in the intertidal and sublittoral zone to a depth of 50.0 m. It will cover its shell into the substrate approximately 2 - 5 cm in depth (Pouteirs, 1998). The posterior side is up close to surface. When the 2 valves of shell are open the siphons will extend from the posterior side. *M. recens* is also filter feeder same as general bivalves. This clam is distributed across the Indo-West Pacific, from the Persian Gulf and India to the Philippines; north to southern Japan and the Taiwan Province of China, and south to Indonesia (Pouteirs, 1998).

1.2.5 Reproductive system of bivalves

Sex and reproduction

The reproductive system in bivalves is extremely simple: the paired gonads are made up of branching tubules, and gametes bud off the epithelial lining of the tubules. Tubules unite to form ducts that lead into larger ducts that eventually terminate in a short gonoduct. In most bivalves, the gonoduct open through the pore into the mantle cavity close to the nephridiopore. Fertilization is external and the gametes are shed through the exhalent opening of the mantle (Gosling, 2003).

The majority of bivalves are dioecious, and there are usually equal numbers of male and females, although the sexes cannot be differentiated by characteristics (Gosling, 2003). Bivalves can be classified into two main groups according to their reproductive system: (1) gonochoristic bivalves that sex are separated e.g. *Cuspidaria* Nado, *Nacula* Lamarck etc. and (2) Hermaphrodite bivalves that have male and female gonad in the same individual e.g. *Ostrea* Linnaeus, *Crassostrea* Sacco, *Anodonta* Lamarck, *Pecten* Muller etc. (Uppatham et al., 1995). In case of *M. recens* is a dioecious/gonochoristic bivalve, but sex identification cannot be done by external character observation. It uses external fertilization by releasing the eggs and sperms into a water column. The gonad of *M. recens* is distributed around its internal organs in the visceral mass.

1.2.6 Gametogenesis and reproductive cycle in bivalves

Morse and Zardus (1997) have described gametogenesis in bivalves like this:

Oogenesis is the gametogenesis in female bivalves, developing oocytes, and usually remaining attached by a stalk to the wall of the follicle throughout development. The oocytes project into the lumen of the follicle as they mature, eventually filling the lumen and taking on irregular shapes as they fill the lumen and are pressed together. Oogenesis starts with primordial germ cells, located within the follicle wall, that develop into primary oogonia; these in turn undergo mitosis to become secondary oogonia, which then undergo meiosis, becoming primary oocytes at the first division, secondary oocytes at the second division, and subsequently differentiating into mature oocytes. Oocytes differentiation includes six phases: germinal vesicle formation, RNA synthesis, vitellogenesis, cortical granule formation, attachment of auxillary cells to the oocyte, and oocyte maturation.

Spermatogenesis, spermiogenesis and sperm are the gametogenesis in male bivalves. Gametogenesis involves the production of spermatogonia by mitotic divisions of primary gonial cells, located along the inner periphery of tubular, secondary follicles that spread into connective tissue. Primary spermatogonia, the largest male germinal cell, are the first cells to differentiate from the inner wall of the follicle. As the development proceeds, the cytoplasm decreases with each cell division, and the cells become smaller and move toward the center of the lumen. During spermatogenesis, spermatogonia undergo mitosis and become spermatocytes; they in turn undergo meiosis and become spermatids. Primary spermatogonia give rise to secondary spermatogonia that have oval nuclei. The latter cells divide and differentiate and become primary spermatocytes. Primary spermatocytes undergo meiosis to produce secondary spermatocytes at the first division and spermatids at the second. The secondary spermatocytes are smaller than secondary spermatogonia and transitory. They are polygonal and spongy-looking. Spermatids, smaller than secondary spermatocytes and irregularly rounded, have a spherical nucleus. Spermiogenesis involves the differentiation of spermatids into spermatozoa without further cell division. The cytoplasm of the spermatids decreases during this phase. The maturing sperm fill the lumen of the follicle with tails and heads aligned in rows.

The study on the reproductive cycle of bivalves using histological analysis to determine the differentiation of gonad (gametogenesis) provides more accurate information about the spawning season than an estimation using general characteristics or the color of the gonad. Therefore, the histological analysis method has been widely used to study the reproductive cycle of many species of molluscs from many countries (Suwanjarat et al., 2008). Serdar and Lok (2009) reported that gametogenic cycle in bivalve species varies from location to location. Geographical location is important in the timing and duration of gametogenesis and spawning. Several studies demonstrate that clams in different geographical areas, with different water temperatures, have one or two spawning seasons. For example, Manila clam *Ruditapes philipinnarum* in Hawaii, which is located close to the equator, spawn all year round with peak spawning periods during December and January (Yap, 1977 cited in Drummond et al., 2006) and similar to the investigation of Kastoro (1995) who found that *Anadara indica* in Jakarta Bay spawn continuously throughout the year. While in Ireland, Northern Europe, Drummond et al. (2006) reported that there was one major spawning period of *R. philippinarum*, from July to September. In the case of Thailand, Suwanjarat et al. (2009) reported that the gametogenesis of *Anadara granosa* in Pattani Bay, southern Gulf of Thailand, occurred throughout the year and also found that environment parameters did not influence the spawning period. Gosling (2003) stated that gametogenesis will cycle according to the season, and this development is related to internal and environmental factors. Generally, the stages of gametogenesis in both males and females bivalves is as follows:

Stage 1 Resting/Prefollicular development: There are many connective tissues in the gonad and germ cells can mix in the connective tissues. Sex differentiation cannot be done at this stage.

Stage 2 Early/Initial development: A small number of first stage male and female gonads occur in the connective tissues.

Stage 3 Late development/ Developing: All stages of gonad development can be distinguished, but the amount is still low.

Stage 4 Ripe/Mature: There are full of spermatozoa in the lumen of the male gonad, and fully mature oocytes are full in the female gonad.

Stage 5 Partially spent/Spawning: There are broken connective tissues inside the gonad due to the release of spermatozoa in males and oocytes in females.

Stage 6 Spent: There are full of broken connective tissues during the early part of this stage. But at the end, the connective tissues start to rearrange. There are some remains of oogonia and spermatogonia.

1.2.7 Reproductive characteristics in bivalves

Condition index

The condition index (CI) is considered the simplest and most practical method to monitor the gametogenic activity in bivalves (Cano et al., 1997; Chavez-Villalba et al., 2007). Various condition indices have been used in bivalve aquaculture e.g. Barraza-Guardada et al., (2009) believed that a CI of Pacific oyster *Crassostrea gigas* postlarvae more than 11.5 is the best represents spat of high quality. Condition indices in aquaculture may serve two purposes. The first is an economic one, in which the index is used to designate the quality of a market product. The second is an ecophysiological one in which the index is used to summarize the physiological activity of the animals (growth, reproduction, secretion, etc.) (Lucas and Beninger, 1985; Cano et al., 1997). Therefore, an index shows the fertility of the clams, coupled with a histological study to clearer explain the results of reproductive biology, because the condition index is related to the gonadal index. The condition index value is considered by the change of clam weight according to the gonad development stage. Bivalve molluscs will intake more food to store as an energy source in order to utilize during reproduction. There are many methods to assess the condition index, but in general this value derives from the weight in different parts of the clam, using the following equations:

$$\mathrm{CI} = \frac{\text{Dry tissue weight (g)}}{\text{Total weight (g)} - \text{Shell weight (g)}}$$ (Tuaycharoen and Prompai, 1991)

$$\mathrm{CI} = \frac{\text{Tissue Weight (g)} \times 1000}{\text{SL (mm)} \times \text{SH (mm) x SW (mm)}}$$ (Choi and Chang, 2003)

$$\mathrm{CI} = \frac{\text{Dry flesh weight (g)} \times 100}{\text{Dry shell weight (g)}}$$ (Walne, 1976 cited in Drummond et al., 2006)

Lucas and Beninger (1985) stated that the “dry flesh weight: dry shell weight” ratio is a widely used condition index, because of the nature of the measurements involved, so can be easily standardized and be more universal. The use of dry tissue weights eliminates any bias due to water content fluctuations of the whole tissue. A low value for this index indicates that a major biological effort has been expended, either as maintenance energy under poor environmental conditions or disease, or in the production and release of gametes. Thus, as an indicator of stress, or sexual activity, this index gives meaningful information about the physiological state of the animal. The condition index of bivalves can act as an indicator of reproductive activity, and the condition of clam meat is dependent on the gametogenic activity. Significant increases in the condition index are seen when the gonad is ripening and gonad growth is favored over somatic growth. The condition index is high just before spawning, and is low immediately after the completion of spawning (Cano et al., 1997). On this basis, it can be inferred that this period is a suitable one for fishing of clams for consumption, due to the presence of meat of high quantity (Suja and Muthiah, 2007).

Ganadal Index

Gonadal index (GI) is one of the most reliable methods for assessing the reproductive cycle in bivalves together with histological of gonad. The gonadal index is derived by multiplying the number of individuals at each development stage by the numerical ranking of that stage, and dividing the result by the total number of individuals in the sample, a mean gonadal index is arrived at for each sampling interval. The gonadal index increases during gametogenesis and decrease during spawning (Gosling, 2003; Idris et al., 2017; Alvarez-Dagnino et al., 2017).

Monthly gonad index can archieved utilizing a numerical grading system to obtain a quantitative value that represents the reproductive activity (Idris et al., 2017). The macroscopic gonad stages of each sample were assigned and gonadal index were calculated according the following formula,

$$GI = \frac{\text{Number in each stage x numerical ranking of that stage}}{\text{Number of calms in the samples}}$$

Generally, the GI values increase during gametogenesis and decrease during spawning and resting. Suwanjarat et al. (2009) stated that GI is the value to estimate the proportion of the gonad development in each stage of bivalve such as resting, developing, mature, spawning and spent individuals. GI is ranks from 1 (all individuals in the sample are in the resting or spent stage) to 3 (all individuals are in the mature stage). GI values of mangrove clam *Polymesoda expansa* in Malaysia indicated that its reproduction was inactive from December to January and gameogenesis started in March and continued until October (Idris et al., 2017). Hamli et al. (2015) found that GI in hard clam, *Meretrix lyrata* correlated with Chlorophyll a suggested that the development of reproductive cycle of hard clam required a high amount of food to increase gametogenesis. Gilbert (1992) cited in Cano et al. (1997) stated that phytoplankton was the best food source for bivalves.

1.2.8 Size at first maturity of bivalves

The size at first maturity of bivalves is the minimum shell length of bivalve that has a gonadal development in the matured stage (Sutthakorn and Tuaycharoen, 1993). King (1995) used the length at 50 percent of aquatic animals in the size class had gonadal development in the matured stage. Jagadis and Rajagopal (2007) stated that monthly percentages of maturity stages plotted against different size group show the size group at which 50% of clams matured. The mean value of this size group is considered as the minimum size at first maturity. Sutthakorn and Tuaycharoen (1993) found that the smallest size at first maturity of *Paphia undulata* (Born, 1778) along the western coast of

Thailand is 1.43 cm. which is different from the Gulf of Thailand such as in Trat province – the size at first maturity is 4.25 cm, while in Suratthani province it is 3.06 cm. Jindalikit (2000) stated that size at first mature of *Paphia undulata* in Mahachai Bay, Samut Sakhon province was 3.16 and 3.19 cm in male and female, respectively while its size at first maturity found in Central Philippines was 4.26 and 4.48 mm in male and female, respectively (Nabuab et al., 2010). The size at first maturity for *Gafrarium tumidum* (Roding, 1798) along the southeast coast of India is 2.23 cm. (Jagadis and Rajagopal, 2007). Kalyanasundaram (1982) cited in Jagadis and Rajagopal (2007) found that the size at first maturity of *Marcia opima* was 2.47 cm.

1.2.9 Environmental factors influencing reproduction in bivalves

The control of reproduction involves the complex interplay of environmental factors such as temperature, food, salinity and light. Temperature is the single exogenous factor that is most often cited as influencing gametogenesis in bivalves (Gosling, 2003). Serdar and Lok (2009) concluded that the completion of the carpet shell clam *Tapes decussates* gametogenesis, seawater temperature was the major factor rather than food availability. Lok et al. (2007) reported that reproduction in marine bivalves is principally affected by a number of environmental factors including water temperature, quantity and quality of food, but phytoplankton availability is the most important. Suwanjarat and Parnrong (1991) found that the varying of seawater temperature is a factor affecting the reproductive of cockle *Anadara granosa* L. in Satun Province on the other hand, the reproductive cycle of this cockle in Pattani Bay, southern Gulf of Thailand did not related to the environment parameters (Suwanjarat et al., 2009). The temperature varying from 25.5 to 30.5 ˚C is suitable for the reproduction of *Meretrix* sp. (Tuaycharoen and Prompai, 1991) and the temperature varying from 25.9 to 33.9 ˚C is suitable for the reproduction of *Donax scortum* (Peerakeitkhachorn, 1995). The gametogenic cycle of pearl oyster, *Pinctada fucata martensii,* shows seasonality, related to the water temperature. Oocytes grow and mature with an increase of water temperature, and oocytes begin release when the temperature rises to 20.1 ˚C. This species has a major peak in spawning activity during summer (Choi and Chang, 2003). The temperature regime is an important factor for the reproduction of scallop *Chlamys nobilis* (Reeve). When these factors are varied, reproduction will vary (Thi Xuan Thu and Chinh, 1999). In case of the salinity, is affect to the reproductive biology of bivalves such as the investigation of Jagadias and Rajagopal (2007) found that the spawning of venus clam *Gafarium tumidum* in the Southeast coast of India was not occurred when the salinity was very low less than 10 ppt and this clams attained peak maturity during the hot season and higher salinities (31.1-34.3 ˚C and above 30 ppt).

1.2.10 The reproductive biology of bivalves: Implications for aquaculture and wild stock management

Extensive commercial exploitation of marine fisheries has led to depletion of numerous fish and shellfish stocks worldwide (Garcia de Severeyn et al., 2000). In fact, a fishery should be governed from the beginning but most of management measures cases are only implemented when overexploitation (overfishing) of the resource is already occurred (Gosling, 2003) The aim of fishery management is to optimize the yield, both in weight and value, and to sustain a particular stock level to provide a cushion against poor recruitment years, or to sustain a minimum spawning stock (King, 1995).

Among biological features, reproductive biology of species is the most widely used for developing management strategies. Determination of first maturity length and spawning period are the basic requirements for the protection and sustainable exploitation of the stocks (Sahin et al., 2006). Detailed knowledge of the reproductive biology of bivalve species, can also provide the optimum reproductive time for artificial spawning induction in aquaculture (Joaquim et al., 2008) and confirmed by Nugranad et al. (2004) that the use of *Meretrix meretrix* brood stocks from nature for breeding, the selection must take place during the mature stage of this clam because mollusks are one of the most important groups in marine aquaculture due to their relatively low cost of production (Bautista, 1989 cited by Garcia de Severeyn et al., 2000). In case of management point of view, the reproductive cycle information is important to obtain a good quality of larvae and consequently to promote successful restocking actions based on aquaculture production and also is relevant to regulate the dredge fishery (Joaquim et al., 2008). The result of the study on reproductive cycle of *Anadara antiquata* by Mzighani (2005) suggested that in order to sustainable fishery management of this cockle, it is recommended that certain periods of time or areas be closed to fishing allowing for this cockle to be recruited and grow out to the maximum size and spawn at least once before harvesting. Nakamura et al. (2010) found that hard clam *Meretrix lusoria* in Ariake Sound and Tokyo Bay, Japan matured at a shell length of 17-20 mm and the minimum size of this clam for fishing is set at a shell length of 33 mm and they also suggested that setting a closing period just before spawning is an effective measure for the protection of populations. Suwanjarat et al. (2009) found that the breeding season of the cockle *Anadara granosa* in Pattani Bay, southern Thailand is mainly from July to August, therefore the conservation of this cockle, exploitation of this cockle should be avoided during July to August.

1.2.11 Geographical characteristic of Tambon Koh Sarai

Tambon Koh Sarai is a subdistrict of Mueang district, Satun Province. It is located on the east coast of Tarutao island, composed of coastal islands surrounded by shallow marine water with an average depth of 1-2 meters at low tide. There are 7 villages in this Tambon: Moo 1 Ban Tanyong-uma, Moo 2 Ban Bagan-yai, Moo 3 Ban Tanyong-kling (these three villages are located in Koh Tanyong-Uma), Moo 4 Ban Yaratod-nui (located in Koh Yaratod-nui), Moo 5 Ban Koh Sarai, Moo 6 Ban Talo-nam (these two villages are located in Koh Sarai) and Moo 7 Ban Koh Lipe which is located in Lipe island, Adang archipelago. The marine area surrounding tambon Koh Sarai has abundant aquatic animals, and for this reason, the main occupation of the villagers is small scale fisheries. The main fishing gears used by local fishermen are crab bottom gillnet, fish gillnet and shell collecting. In the case of shell collecting, most of the fishermen collect for home consumption or for sale in the village. The target species of clam that is abundant in the area is short-neck clam, *Marcia recens* (Holten, 1802), locally called “Hoi Ka-yam”. All coastal areas in Tambon Koh Sarai are natural habitat of short-neck clam, composed of sand or muddy-sand substrata. The fishing grounds of short-neck clam are shown in Figure 2.

The upper fishing ground located between Koh Pulao-O and Ban Bagan-yai (on the west of Koh Tanyong-uma) is used by the fishermen of Ban Bagan-yai and Ban Tanyong-uma. The lower fishing ground is the main fishing ground for people from Ban Koh Sarai, Ban Tanyong-kling, Ban Yaratod-nui, Ban Talo-nam and also for fishermen outside Tambon Koh Sarai, from Ban Thung-rin, Tambon Sakorn, Thapae district. Normally, local fishermen in Tambon Koh Sarai collect for short-neck clam by hand collecting on the muddy flat during the low spring tide. The fishermen from Ban Thung-rin, village nearby Tambon Koh Sarai collect this clam by using a hand dredge, which can operate in deeper waters, so they can collect short-neck clam everyday independently of the tides. The local fishermen in Tambon Koh Sarai believe that dredging can cause the rapid decline of the short-neck clam stock at Tambon Koh Sarai.

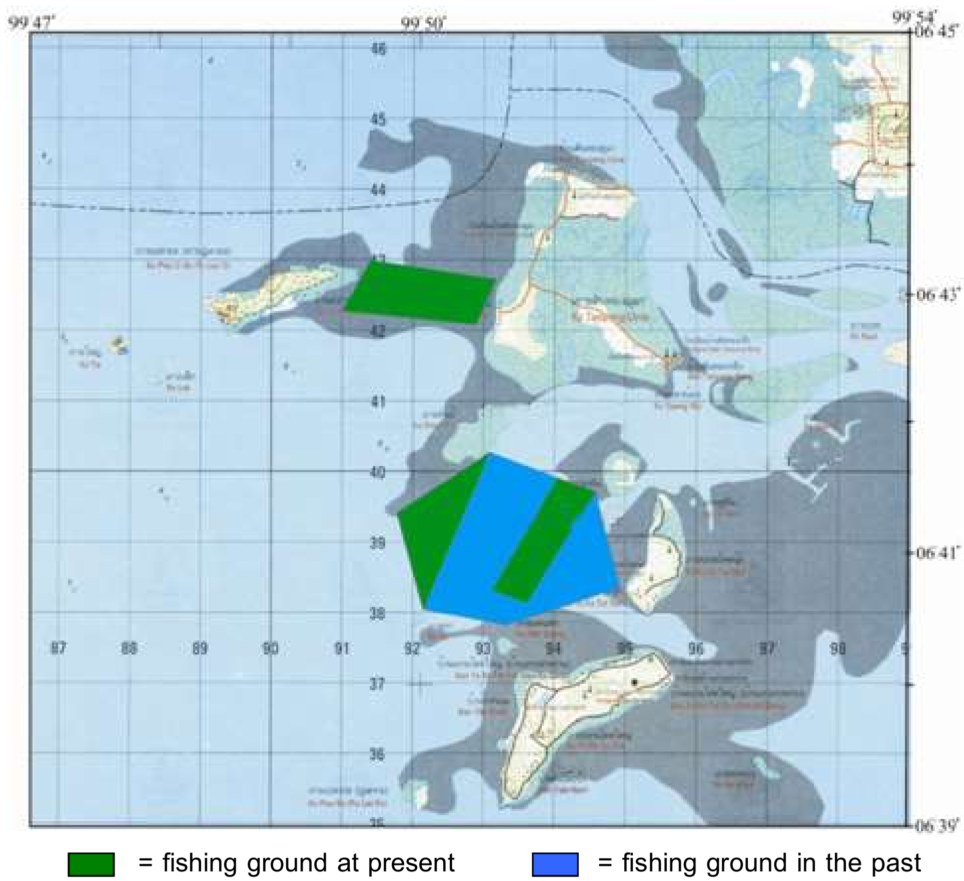

Figure 2 Map of the fishing grounds of short-neck clam *Marcia recens* (Holten, 1802) in Tambon Koh Sarai.

1.3. Objectives

1. To study the reproductive cycle of *Marcia recens* (Holten, 1802) at Tambon Koh Sarai.

2. To study some selected reproductive characteristics including the size at first maturity, sex ratio, condition index and gonadal index of *Marcia recens* (Holten, 1802).

3. To study environmental parameters in the habitats of *Marcia recens* (Holten, 1802), including the general characteristics of the area, water quality (salinity, temperature, pH and Dissolved Oxygen), chlorophyll a and organic matters in soil.

CHAPTER 2

Materials and Methods

2.1 Sampling Sites

Marcia recens samples were taken from two sites in the area of Tambon Koh Sarai, Satun Province, Southern Thailand. The first site was near Koh Plao Yan (N 06˚ 41.374', E 99˚ 51.083') and the second was at Chong Lad (N 06˚ 42.168', E 99˚ 50.436'). The distance between the two sites is approximately 2 km. (Fig. 3). The sites are both tidal flat areas with similar physical characteristics such as a sandy substrate, water level, and distance from land and the influences of human activity.

2.2 Sampling Method

Samples were taken using a 1 m^2 quadrat, placed on the substrate during the lowest spring tide during a new moon. The clams were handpicked at depths of 5 - 10 cm. At least ten 1 m^2 quadrat samples were gathered monthly at each site: at the Pao Yan site between November 2009 and February 2011, and at Chong Lad between March 2010 and February 2011. The clams were transported live back to the laboratory of Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkla University for reproductive biology studies of *Marcia recens*.

Measurements of shell length (SL) (Fig. 4) were carried out with a vernier caliper accurate to the nearest 0.01 mm. The shell lengths were categorized into four size classes: 10.00 - <20.00 mm, 20.00 - <30.00 mm, 30.00 - <40.00 mm, and 40.00 - <50.00 mm, and the number of clams in each class were recorded. Individual weights were recorded with a precision balance (a Sartorius B3100S) to the nearest of 0.01 g.

Twenty individuals were selected from each size class, and the resulting 80 clams were measured again for shell height (SH), and shell width (SW) (Fig. 4). Forty individuals were used for a histological study, and the others in a condition index study.

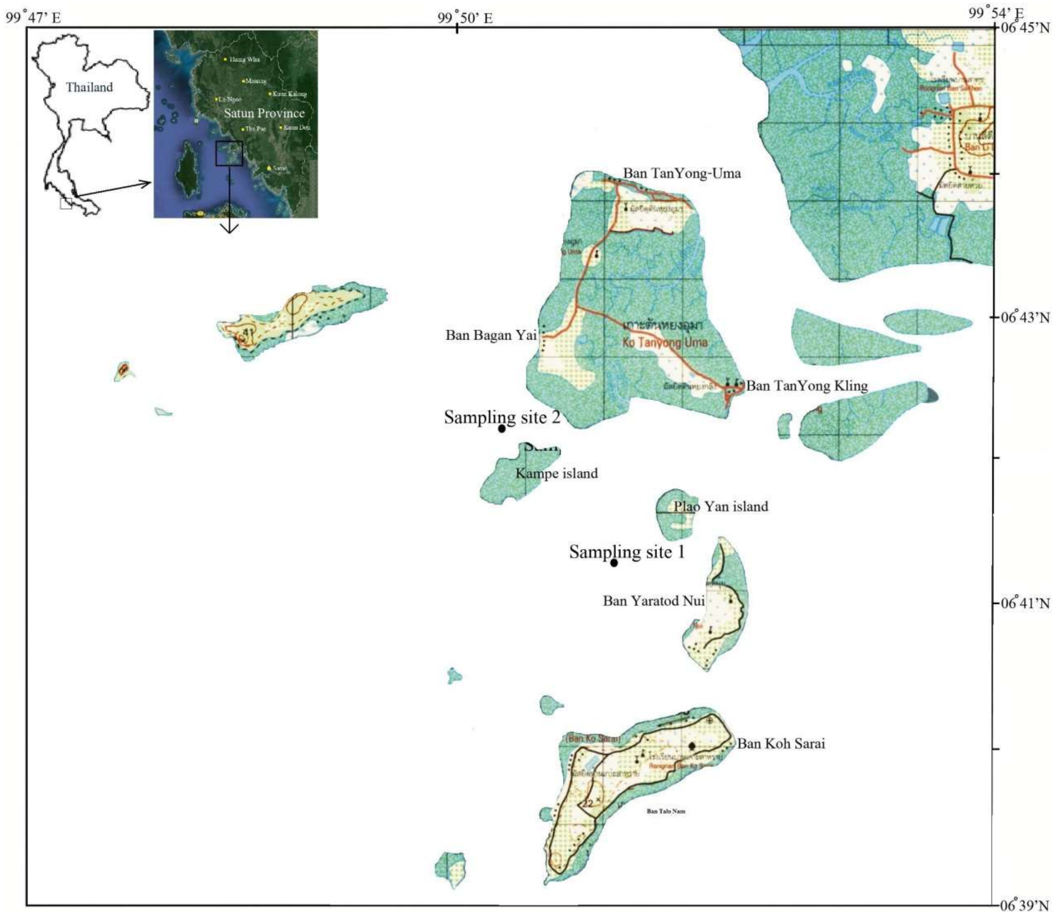

Figure 3 Sampling sites at Tambon Koh Sarai, Satun Province, southern Thailand.

Figure 4 Shell measurements of *Marcia recens.*

2.3 Environmental parameters recording

Environmental parameters were recorded during clam sampling. The water temperature, salinity and pH at each sampling site were noted 0.5 m below the water surface using a mercury thermometer, a handle salino-refractometer (ATAGO, S28) and a pH meter (Metter MP-120). Three water samples were collected using 250 ml BOD bottles and 1,000 ml plastic bottles at each site. Dissolved Oxygen (DO) and Chlorophyll *a* were analyzed using the Azide modification method and the Trichromatic method, respectively (APHA et al., 1995).

Three soil samples were taken at each sampling site using an acrylic four cm diameter corer. The corer was pushed ten centimeters down into the substrate. The percentages of organic matters were estimated following the Walkley and Black method (Nelson and Sommers, 1982). The soil texture (i.e. the percentage of sand, silt, and clay) was determined using the standard hydrometer method (Gee and Bauder, 1986)

2.4 Histological Study

Tissue Preparation

The soft tissues of the clams were removed from their shells, following by cutting each longitudinally in half, to provide a transverse section containing the gonad, digestive gland, and muscular foot tissue of each half. The specimens were fixed in neutral buffered formalin fixative (pH 7.00) (Drury and Wallington, 1980) for 24 hours using the Humason method (1972). The samples were washed in running water for 30 minutes and stored in 70% ethyl alcohol for at least 24 hours. Subsequently, they were dehydrated by being immersed in a graded series of ethanol, dealcoholised in xylene, and embedded in paraplast using an automatic tissue processor (Technical®) and tissue embedding center (Technical®). Paraplast blocks were sectioned into 5 µm thicknesses using a rotary microtome, and stained with Harri's haematoxylin (ACTA®) and counter-stained in eosin (Bancroft and Gamble, 2002). The prepared slides were examined using x5, x10 and x40 magnifications under a light microscope (an Olympus BX 50), and photographs taken with an Olympus PM-10AD, Nikon DS-U2, to determine sex and stage of reproductive development.

Reproductive Development investigation

Reproductive maturity was identified following Drummond et al. (2006) which categorizes reproductive development into six stages:

Stage 0 (Resting Stage): the gonad is predominantly composed of connective tissue, and sexes cannot be differentiated.

Stage 1 (Early Development Stage): gonad proliferation starts; in males follicles compose of many follicle cells, spermatogonia is developing nearby the follicle walls, spermatocytes can distinguish, but spermatids or spermatozoa are not present; in females: an increasing number of discernible oocytes are in the follicle walls; oocytes are small; there are no free oocytes present in the center of the follicle.

Stage 2 (Late Development stage): in males: spermatogonia, spermatocytes, spermatids and spermatozoa are present in the follicles; in less developed gonads dominant cell type cannot be observed, but in more developed gonads, Most of follicles are full of spermatids and spermatozoa; in females: there are free oocytes present in the center of follicles but the amount is less than half of the total oocytes present in the follicles; attached oocytes are equally abundant.

Stage 3 (Ripe Stage): in males: the follicles are mainly composed of matured spermatozoa with their flagellum pointing towards the center of the follicle, to form concentric bands or plugs; In very ripe specimens, spermatozoa bands are close to the follicle wall; the appearance of follicles are neat and orderly; in females: the gonad occupies a large surface area, Many oocytes are free in the center of follicles, The shape of follicles is in a polygonal configuration and their walls are thin.

Stage 4 (Partially Spent Stage): in males: spermatozoa is clearly visible in a swirling shape and account for the greatest portion of cells in the follicle; there is empty space in some follicles due to the release of mature spermatozoa; in females: free oocytes in each follicle are reduced; some follicles are empty due to the releasing of gametes; the follicle walls are broken.

Stage 5 (Spent or Resorbing Stage): the follicles appear broken, scattered, and relatively empty; in males: in advanced spent individuals, only residual spermatozoa are found and are undergoing resorption; there is a presence of phagocytes; in females: only residual oocytes can be found in the follicles, with most of them areundergoing resorption; Many phagocytes are present.

When more than one development stage is evident within a single individual, the clam is assigned to the reproductive stage that is observed in the majority of the follicles.

2.5 Gonadal Index Study

The gonadal index provides an estimation of the reproductive activity among individuals in a population. After the observation the slide of gonad tissue of the short-neck clam under the light microscope in oreder to differentiate the sex of clam and the stage of its gonad. Six reproductive stages are assigned numerical scores: Resting = 0, Early Development = 3, Late Development = 4, Ripe = 5, Partially Spent = 2, and Spent = 1 (Gosling, 2003). A mean gonad index (GI) of short-neck clams for the sampling month is calculated using the method proposed by Gosling (2003). The GI values range from 0 (all individuals are in the resting stage) to 5 (all the individuals are ripe).

$$\mathrm{GI} = (\sum_{i=0}^{5} \mathrm{n_i R_i})/\mathrm{N}$$

When GI = gonadal index

$\mathrm{n_i}$ = number of individuals from each development stage i

$\mathrm{R_i}$ = ranking number of each development stage i

N = total number of clam ineach sampling month

2.6 Sex Ratio

The sexes of short-neck clam cannot be distinguished from an examination of their external characteristics. Instead, the presence of oocytes or spermatozoa in the monthly tissue sections is used to differentiate male and female in this study. The test hypothesis for this study is whether the sex ratio of short-neck clam is equal to 1:1, as found in other bivalves.

2.7 Size at first maturity of *Marcia recens* (Holten, 1802)

The reproductive development stages of *Marcia recens* can be divided into two groups: immatured and matured. Immatured *Marcia recens* is defined as those clams with their reproductive development stage in resting, early, or late development. Matured *Marcia recens* clams are in the reproductive development stages of ripe, partially spent, or spent.

The relationship between size and developmental stages is calculated based on an equation developed by Somerton (1980). The shell lengths are divided into seven classes: 15.00 - <20.00, 20.00 - <25.00, 25.00 - <30.00, 30.00 - <35.00, 35.00 - <40.00, 40.00 - <45.00 and 45.00 – <50.00 millimeters. The relationship between the possibility of mature *Marcia recens* and their shell length or mid length can be expressed as:

$$y = 1/(1 + e^{(a+bx)})$$ (Somerton, 1980)

When y = possibility of being a matured *Marcia recens*

x = the mid length of the shell length class interval

a and b = parameters derived from simple linear regression equation

2.8 Condition Index study

The flesh tissues of 40 clams from each sampling site were removed from their shells and dried at 60 °C for 48 hours in incubator. Individual fresh and dry weights of both tissue and shells were obtained using a analytical balance (a Sartorius R200D). The condition index (CI) was calculated according to Walne (1976 cited in Suja and Muthiah, 2007) as:

$$\mathrm{CI} = \frac{\text{dry flesh weight (g) x 100}}{\text{dry shell weight (g)}}$$

2.9 Statistical Analysis

The Pearson product-moment correlation was used to examine the correlations between male and female reproductive cycles and environmental parameters such as temperature, salinity, pH, DO, chlorophyll *a* and organic matter in soil together with condition index, and gonadal index. A Chi-square test was used to analyze the sex ratios between both sampling sites. Unpaired *t*-test was used to examine the difference of some environmental parameters between the sampling sites

CHAPTER 3

Results

3.1 Environmental Parameters

The seawater temperature at Plao Yan ranged between 27 ˚C and 31 ˚C. The lowest temperature was recorded in December 2010 and the highest in May 2010, with an average of 29 ˚C. The temperature at Chong Lad was similar to that in Plao Yan over the sampling period, reaching a high of 32 ˚C in April and May 2010, and a low of 26 ˚C in January 2011. The average temperature over the sampling period was 29 ˚C (Fig. 5). There was no significant difference between temperatures at the two sampling sites (Unpaired *t*-test, t = 0.879, d.f. = 26, P = 0.502).

Salinity at Plao Yan fluctuated between 21 and 36 ppt. The lowest salinity was found in November 2009 and the highest in March 2010. Salinity at Chong Lad followed a pattern similar to Plao Yan, fluctuating between 26 ppt and 33 ppt. The lowest salinity was recorded in December 2010 and the highest in March 2010. The salinity was generally higher during the hot season (October-April) and lower in the rainy season (May-September) (Fig. 6). There was no significant difference between salinities at the two sampling sites (Unpaired *t*-test, t = 0.421, d.f. = 26, P = 0.811).

The water was slightly alkaline at both sites. The seawater pH at Plao Yan fluctuated between 7 and 8, with the lowest value found in June 2010 and the highest in December 2010. The pH at Chong Lad fluctuated between 7 and 8 and followed a pattern similar to Plao Yan (Fig. 7). There was no significant difference between pH at the two sampling sites (Unpaired *t*-test, t = 0.230, d.f. = 26, P = 0.627). The analysis showed that pH from both sampling sites was significantly negative correlated with temperature. (The Pearson product-moment correlations were r = -0.602, n = 16, P = 0.014 for Plao Yan and r = -0.700, n = 12, P <0.011 for Chong Lad,).

Dissolved Oxygen (DO) at Plao Yan ranged between 5.33 and 7.28 mg/l. The lowest DO was recorded in April 2010 and the highest in November 2010. DO values at Chong Lad followed a similar pattern to Plao Yan, ranging between 5.08-6.68 mg/l. The lowest DO was measured in June 2010 and the highest in April 2010 (Fig. 8). There was no significant difference between DOs at the two sampling sites (Unpaired *t*-test, t = 0.191, d.f. = 26, P = 0.253).

Chlorophyll *a* at Plao Yan ranged between 1.91 and 8.24 µg/l, with the lowest value found in February 2011 and the highest in May 2010. The chlorophyll *a* value at Chong Lad fluctuated between 1.12 µg/l and 3.10 µg/l. The lowest chlorophyll *a* value was recorded in September 2010 and the highest in October 2010. The chlorophyll *a* values at Plao Yan were generally higher than those at Chong Lad (Fig. 9). Plao-Yan experienced significantly higher Chlorophyll *a* than Chong-Lad (Unpaired *t*-test, t = 0.036, d.f. = 18, P = 0.000) and the analysis showed that chlorophyll *a* at Plao Yan was significantly positive correlated with temperature (The Pearson product-moment correlation was r = 0.687, n = 10, P = 0.028).

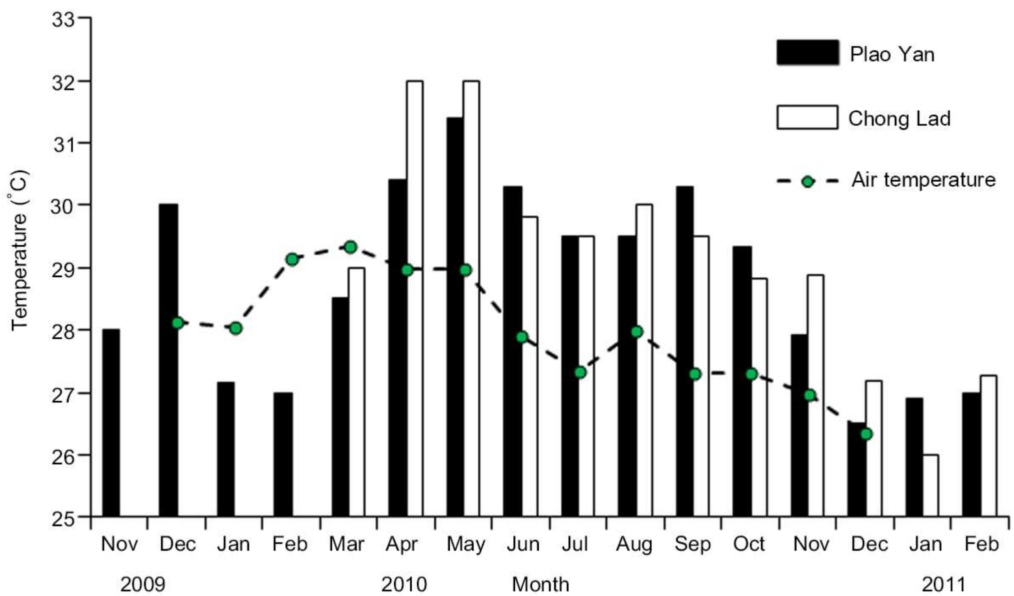

Figure 5 Seawater temperature at Plao Yan between November 2009 and February 2011, and at Chong Lad between March 2010 and February 2011.

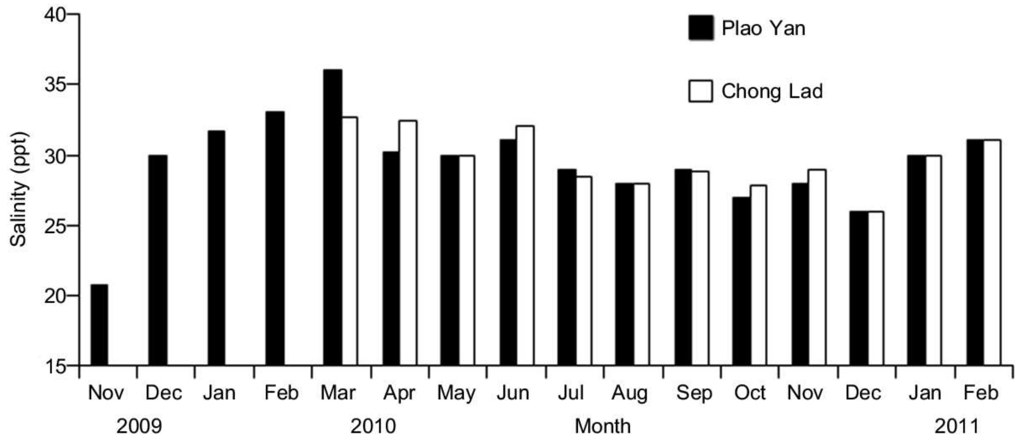

Figure 6 Salinity at Plao Yan between November 2009 and February 2011, and at Chong Lad between March 2010 and February 2011.

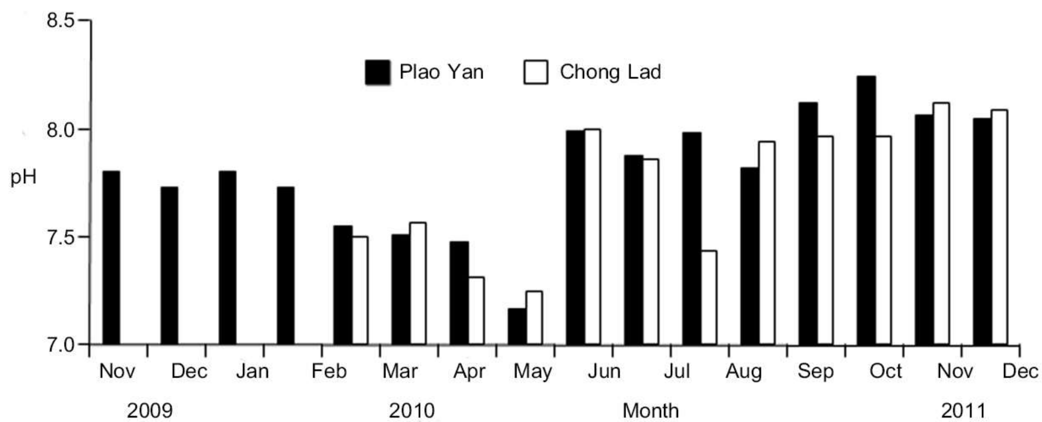

Figure 7 pH values at Plao Yan between November 2009 and February 2011, and at Chong Lad between March 2010 and February 2011.

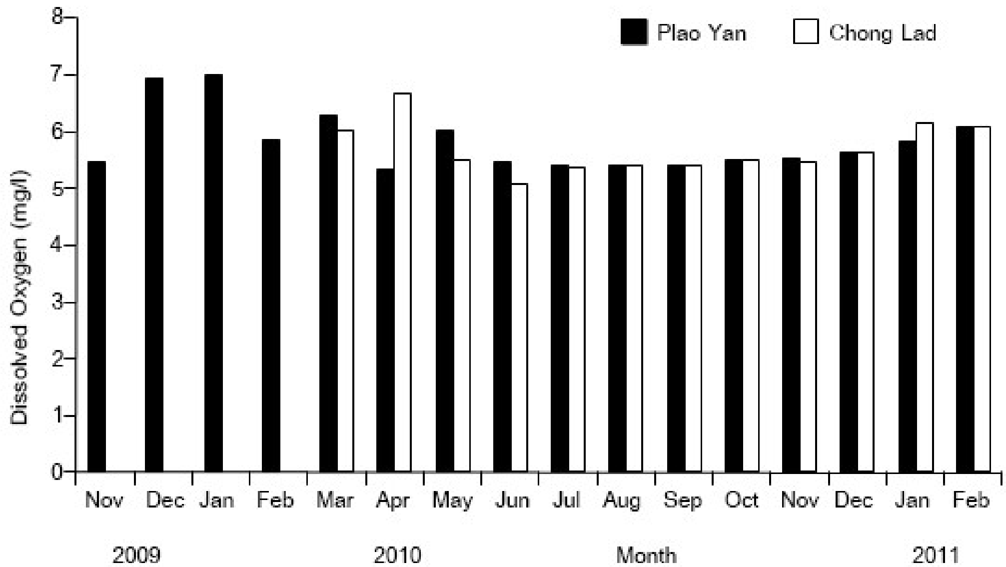

Figure 8 Dissolved Oxygen at Plao Yan between November 2009 and February 2011, and at Chong Lad between March 2010 and February 2011.

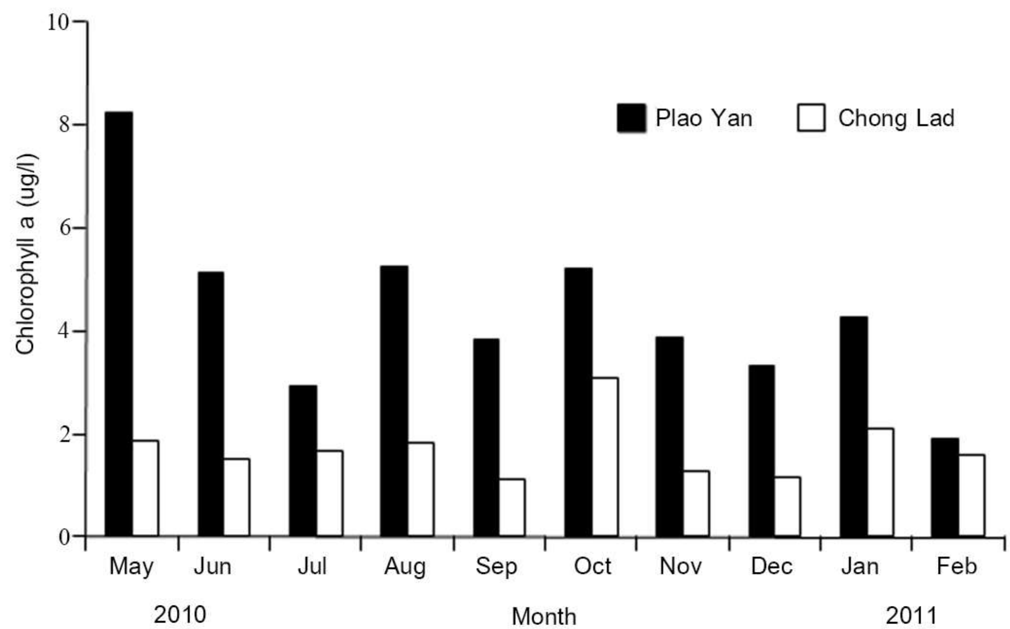

Figure 9 Chlorophyll *a* values obtained from monthly water samples collected at Plao Yan and Chong Lad between May 2010 and February 2011.

3.2 Soil properties in the habitat of *Marcia recens* (Holten, 1802)

Organic matter percentages in the soil at Plao Yan ranged between 0.98% and 1.84%. The lowest value was measured in October 2010 and the highest in November 2009. At Chong Lad, the organic matter percentages ranged between 0.67% and 1.33%, with the lowest value recorded in September 2010 and the highest in August 2010 (Fig. 10).

The soil characteristic of the short-neck clam habitat at Plao Yan is sandy clay loam, with a particle size with diameter less than 2 mm. The monthly average percentages of sand, silt and clay varied between 36.25% and 75.31%, 7.16% and 32.97% and 17.54% and 32.09%, respectively (Table 1). The soil characteristic at Chong Lad is sandy loam, and its monthly percentages of sand, silt and clay varied between 48.09% and 74.67%, 7.21% and 34.69% and 17.00% and 22.94%, respectively (Table 2).

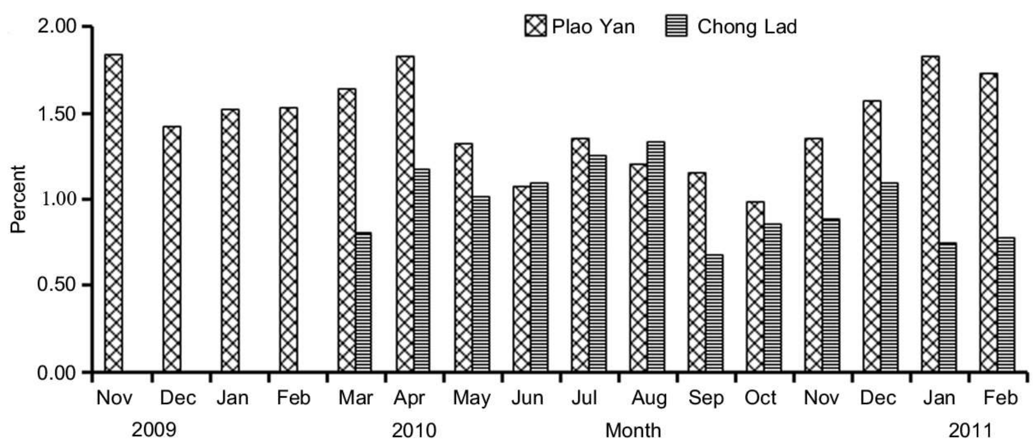

Figure 10 Percentages of organic matter in the soil at Plao Yan between November 2009 and February 2011, and at Chong Lad between March 2010 and February 2011.

Table 1 The monthly organic matter percentages in the soil, and the soil texture, for the *Marcia recens* (Holten, 1802) habitat at Plao Yan between November 2009 and February 2011.

Month	% ± SD				Soil texture
	OM	Sand	Silt	Clay	
November 2009	1.84 ± 0.35	70.37 ± 4..38	7.49 ± 0.62	22.13 ± 3.98	Sandy clay loam
December	1.42 ± 0.02	72.52 ± 1.60	8.49 ± 0.61	18.99 ± 1.04	Sandy loam
January 2010	1.52 ± 0.16	75.31 ± 3.35	7.16 ± 1.75	17.54 ± 1.65	Sandy loam
February	1.53 ± 0.20	67.56 ± 2.53	11.19 ± 1.06	21.25 ± 1.47	Sandy clay loam
March	1.64 ± 0.13	65.96 ± 3.24	11.04 ± 0.55	23.00 ± 2.81	Sandy clay loam
April	1.83 ± 0.08	49.72 ± 2.70	21.31 ± 3.16	28.96 ± 0.49	Sandy clay loam
May	1.32± 0.22	52.06 ± 3.21	21.53 ± 1.59	26.41 ± 2.45	Sandy clay loam
June	1.07 ± 0.28	61.31 ± 8.30	16.69 ± 4.54	22.00 ± 4.21	Sandy clay loam
July	1.35 ± 0.15	51.00 ± 3.42	25.08 ± 1.24	23.93 ± 3.19	Sandy clay loam
August	1.20 ± 0.11	53.78 ± 3.46	23.32 ± 3.11	22.91 ± 2.14	Sandy clay loam
September	1.15 ± 0.16	45.28 ± 2.94	31.27 ± 3.38	23.44 ± 2.19	Loam
October	0.98 ± 0.12	46.58 ± 8.37	32.97 ± 6.16	20.45 ± 2.25	Loam
November	1.35 ± 0.11	49.29 ± 7.22	27.27 ± 8.06	23.45 ± 2.23	Loam
December	1.57 ± 0.08	41.53 ± 0.21	29.72 ± 2.71	28.76 ± 2.51	Clay loam
January 2011	1.83 ± 0.11	36.25 ± 2.61	31.66 ± 2.76	32.09 ± 2.16	Clay loam
February	1.73 ± 0.21	40.55 ± 0.41	28.57 ± 2.08	30.88 ± 2.48	Clay loam

Table 2 The monthly organic matter percentages in the soil, and the soil texture, for the *Marcia recens* (Holten, 1802) habitat at Chong Lad between March 2010 and February 2011.

Month	% ± SD				Soil texture
	OM	Sand	Silt	Clay	
March 2010	0.80± 0.15	74.67± 7.79	7.21± 1.21	18.12±6.61	Sandy loam
April	1.17± 0.14	48.09±15.95	34.69±19.21	17.23±9.96	Loam
May	1.01± 0.19	61.57± 7.77	15.49± 5.83	22.94±1.94	Sandy clay loam
June	1.09± 0.19	56.19± 5.89	24.60± 5.91	19.21±4.16	Sandy loam
July	1.25± 0.20	55.46± 5.73	21.93± 3.18	22.61±3.23	Sandy clay loam
August	1.33± 0.28	48.92± 4.60	29.14± 2.25	21.94±3.45	Loam
September	0.67± 0.15	58.17± 7.27	24.83± 3.43	17.00±3.89	Sandy loam
October	0.85± 0.21	54.21± 7.34	27.97± 3.96	17.82±3.38	Sandy loam
November	0.88± 0.15	53.72± 7.25	27.52± 7.55	18.76±2.18	Sandy loam
December	1.09± 0.47	63.48± 1.70	16.88± 0.96	19.64±2.09	Sandy loam
January 2011	0.74± 0.15	65.43± 3.12	15.85± 2.61	18.72±1.12	Sandy loam
February	0.77± 0.15	64.50± 2.85	16.61± 1.67	18.89±2.17	Sandy loam

3.3 Density of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

Monthly density of *Marcia recens* at Plao Yan was ranged between 7.47 and 26.40 individuals/m^2. The lowest density was found in November 2010 and the highest density was found in February 2010 (Fig. 11). The average density of the *Marcia recens* over the course of sampling period was 13 individuals/m^2. The monthly density of the *Marcia recens* at Chong Lad was ranged between 8.96 and 26.33 individuals/m^2. The lowest density was found in March 2010 and the highest density was found in November 2010 (Fig. 12). The average density of the clams over the course of sampling period was 18 individuals/m^2.

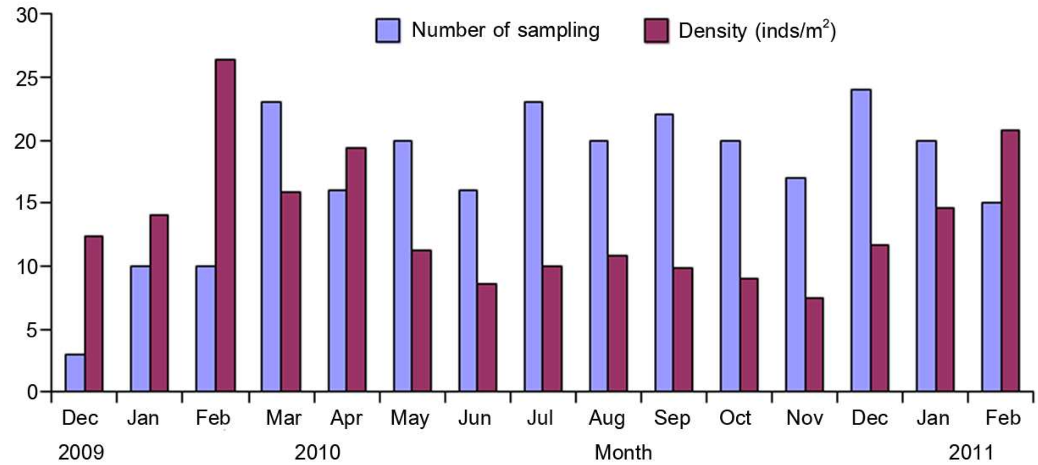

Figure 11 Number of sampling and density of *Marcia recens* (Holten, 1802) at Plao Yan between December 2009 and February 2011.

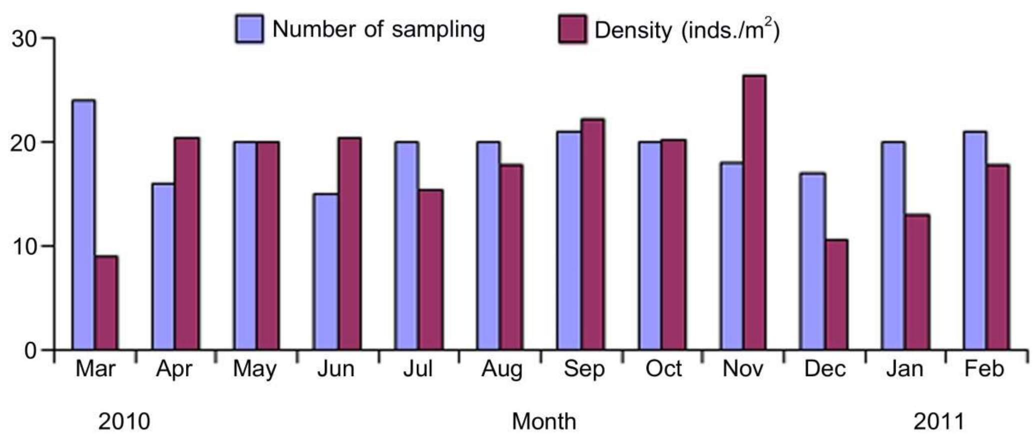

Figure 12 Number of sampling and density of *Marcia recens* (Holten, 1802) at Chong Lad between March 2010 and February 2011.

3.4 Size and weight distribution of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

Marcia recens were sampled from both sampling sites, Plao Yan and Chong Lad in total of 6,982 individuals. 3,607 individuals were sampled from Plao Yan and 3,375 individuals were sampled from Chong Lad (Table 3 and 4). The average shell length of these clams at Plao Yan was 34.74 ± 6.03 (10.15 - 48.70) mm and the average total weight was 11.60 ± 6.09 (0.27 - 39.08) g. The maximum shell length was found in October 2010 and the minimum shell length was found in July 2010, while the maximum total weight was found in June 2010 and the minimum total weight was found in October 2010.

Table 3 The average shell length (SL) and total weight (TW) of *Marcia recens* (Holten, 1802) sampled from Plao Yan (mean ± SD)

Month	No.	SL (mm)			TW (g)		
		mean ± SD	max.	min.	mean ± SD	max.	min.
Nov 09	90	33.77 ± 3.29	41.35	25.55	9.59 ± 3.29	18.26	3.58
Dec	217	33.50 ± 6.59	48.00	13.70	9.73 ± 6.10	37.70	0.41
Jan 10	140	31.90 ± 6.03	44.60	18.00	8.44 ± 5.37	25.75	1.00
Feb	264	33.81 ± 5.56	47.70	15.60	10.15 ± 4.97	24.09	0.63
Mar	364	33.86 ± 4.91	43.80	15.20	10.23 ± 4.56	25.13	0.61
Apr	311	34.80 ± 4.24	45.30	14.90	11.14 ± 4.21	30.35	0.67
May	224	36.30 ± 6.16	45.80	13.20	13.37 ± 5.63	27.55	0.35
June	138	39.34 ± 5.41	48.15	16.85	17.79 ± 6.50	39.08	0.88
Jul	231	35.77 ± 7.89	47.90	10.15	13.75 ± 7.95	34.76	0.93
Aug	216	35.00 ± 7.20	46.50	17.20	12.18 ± 7.05	26.48	0.92
Sep	218	37.54 ± 6.71	46.75	16.85	14.88 ± 7.00	29.20	0.75
Oct	180	33.23 ± 8.26	48.70	12.20	16.65 ± 7.40	30.35	0.27
Nov	127	37.27 ± 6.16	47.25	15.95	14.53 ± 6.63	27.29	0.69
Dec	282	34.84 ± 4.70	46.20	16.35	11.38 ± 5.55	28.89	0.83
Jan 11	293	33.41 ± 4.50	42.90	19.50	10.35 ± 4.84	26.22	1.36
Feb	312	33.84 ± 4.74	45.95	19.05	10.61 ± 4.53	25.00	1.31

The average shell length of these clams at Chong Lad was 34.25 ± 5.60 (12.25 - 48.50) mm and the average total weight was 10.32 ± 5.17 (0.82 - 27.48) g. The maximum and minimum shell length was found in July 2010, while the maximum total weight was found in November 2010 and the minimum total weight was found in July 2010.

Table 4 The average shell length (SL) and total weight (TW) of *Marcia recens* (Holten, 1802) sampled from Chong Lad (mean ± SD)

Month	No.	SL (mm)			TW (g)		
		mean ± SD	max.	min.	mean ± SD	max.	min.
Mar	139	36.58 ± 4.13	46.20	20.80	12.84 ± 4.56	27.10	2.26
Apr	218	36.55 ± 3.64	46.00	25.90	12.33 ± 4.02	21.07	3.30
May	360	36.78 ± 3.27	45.10	26.70	12.82 ± 3.81	25.13	3.49
June	307	37.24 ± 3.53	44.80	18.00	12.60 ± 3.62	25.30	0.97
Jul	306	33.56 ± 8.51	48.50	12.25	10.86 ± 6.72	25.50	0.82
Aug	356	29.21 ± 6.80	47.10	14.90	6.16 ± 4.97	23.75	0.49
Sep	466	32.54 ± 5.71	45.60	18.35	8.42 ± 5.19	25.45	0.98
Oct	403	34.11 ± 4.73	45.40	20.10	9.82 ± 5.15	26.93	1.44
Nov	474	35.97 ± 3.62	44.90	22.05	11.90 ± 4.48	27.48	3.03
Dec	181	32.88 ± 4.12	43.20	19.90	8.47 ± 3.50	19.65	1.33
Jan 11	259	32.88 ± 4.10	41.90	19.50	8.75 ± 3.81	20.26	1.32
Feb	263	34.66 ± 3.60	42.50	17.00	10.26 ± 3.63	20.86	0.86

The comparison of average shell length (SL) (Fig. 13) and average total weight (Fig. 14) of *M. recens* between 2 sampling sites was not different.

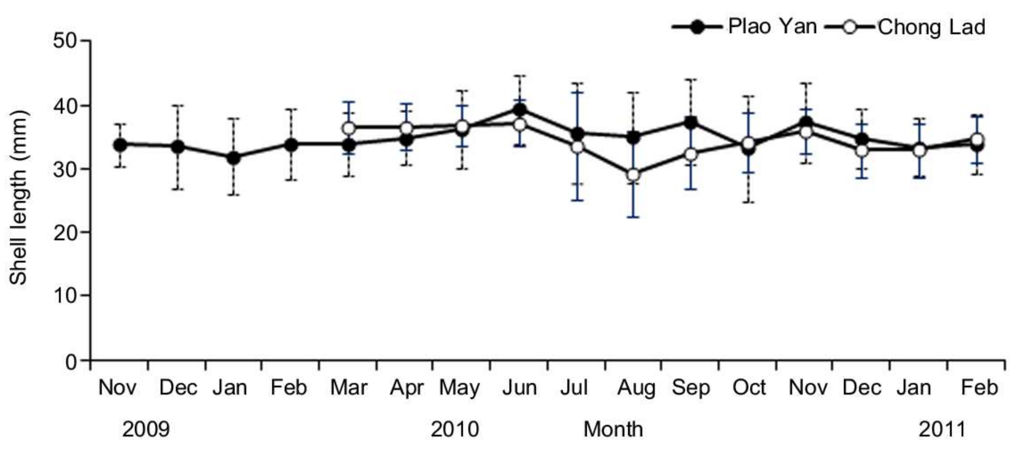

Figure 13 The average shell length (mean ± SD) of *Marcia recens* (Holten, 1802) in each sampling month between November 2009 and February 2011.

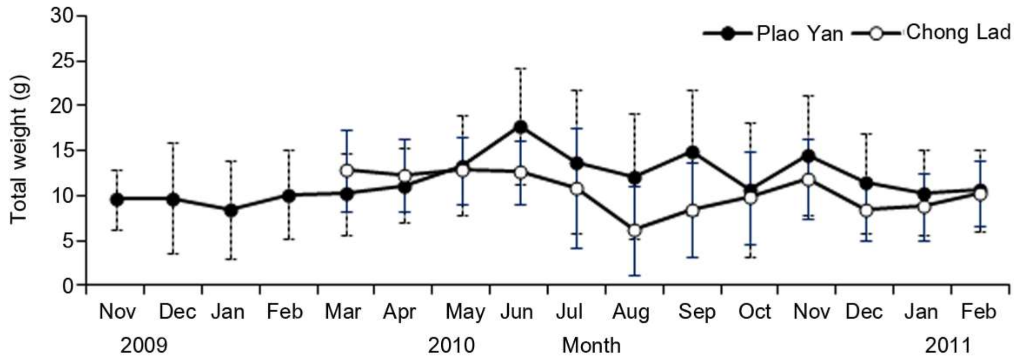

Figure 14 The average total weight (mean ± SD) of *Marcia recens* (Holten, 1802) in each sampling month between November 2009 and February 2011.

3.5 Sex ratio and size at first maturity of *Marcia recens* (Holten, 1802)

3.5.1 Sex ratio of *Marcia recens* (Holten, 1802)

All of the 1,177 clams examined, 540 were male and 580 were female while 44 were sexually undifferentiated (Fig. 15A) which their shell lengths varied between 13.20 and 25.00 millimeters, and also found 3 hermaphrodites (Fig. 15B) in which most of follicles in the gonad were females while a few follicles were males. The overall male/female sex ratio of 1:1.07 showed very similar to a 1:1 ratio (Table 5 and Fig. 16). In case of Plao Yan, The total of 701 clams examined. 333 were male and 337 were female while 23 were sexually undifferentiated which their shell length varied between 13.20 and 23.20 mm, 3 were hermaphrodites and 5 were damaged during the histological preparation. The male/female sex ratio of 1:1.01 showed not different from a 1:1 ratio (Table 5). The total of 476 clams at Chong Lad were examined comprised 207 males and 243 females while 21 were sexually undifferentiated which their shell length varied between 14.20 and 25.00 mm and 5 were damaged during the histological preparation. Hermaphrodite was not found at this sampling site. The male: female sex ratio for Chong Lad was 1:1.17 which was not different from a 1:1 ratio but comparing with Plao Yan, female individuals were higher. Undifferentiated specimens usually occurred in the clams smaller than 25.00 mm shell length.

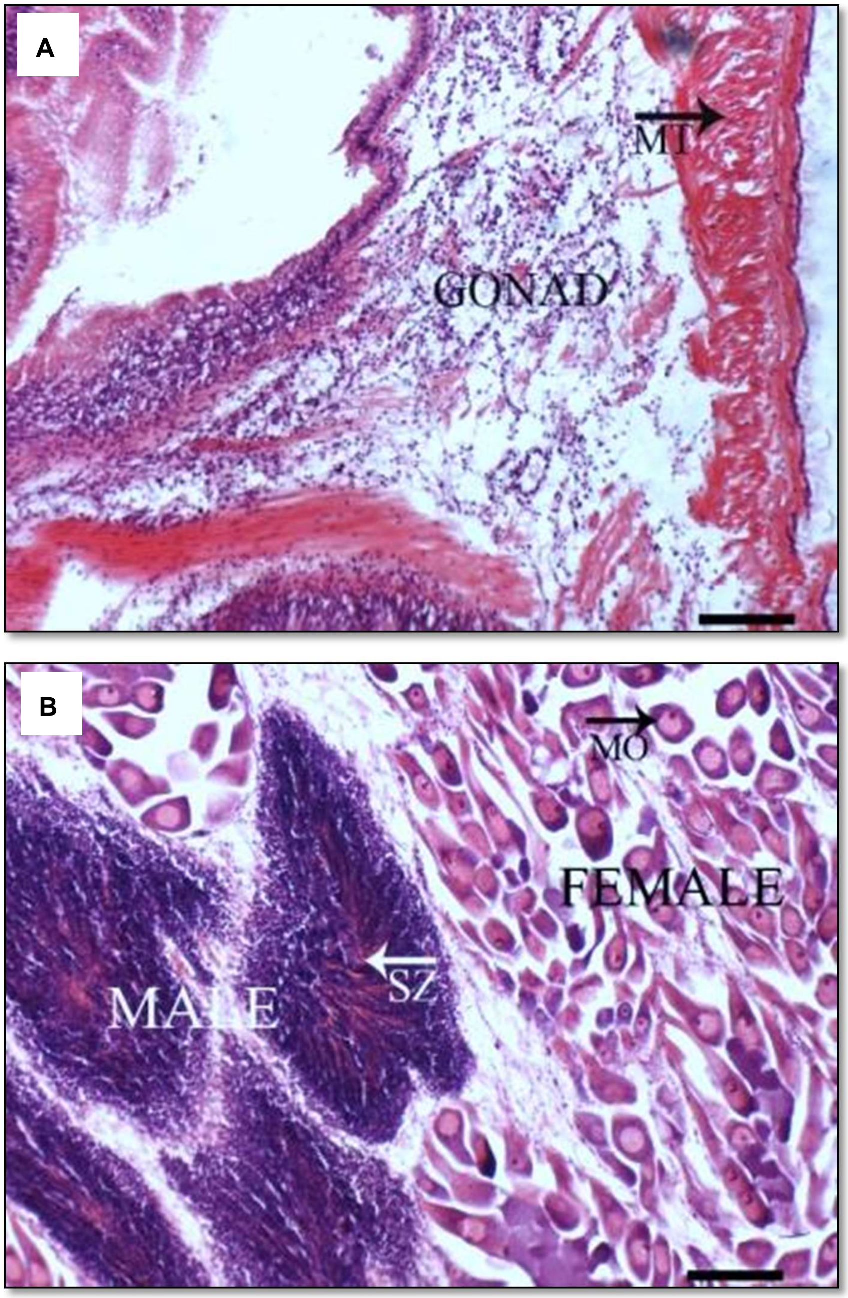

Figure 15 photomicrographs of *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: A = sexually undifferentiated, B = Hermaphrodite; MT = muscular tissue, MO = mature oocyte, SZ = spermatozoa; Scale bar = 100 μm.

Table 5 Numbers and percentage of males, females, sex ratio, sexually undifferentiated and hermaphrodite *Marcia recens* (Holten, 1802) in each sampling site

Sampling site	Male	Female	Sex ratio Male : female	Sexually undifferentiated	Hermaphrodite	Total
Plao Yan	333 (28.53%)	337 (28.88%)	1 : 1.01	23 (1.97%)	3 (0.26%)	696 (59.64%)
Chong Lad	207 (17.74%)	243 (20.82%)	1 : 1.17	21 (1.80%)	0 (0%)	471 (40.36%)
Total	540 (46.27%)	580 (49.70%)	1 : 1.07	44 (3.77%)	3 (0.26%)	1,167 (100%)

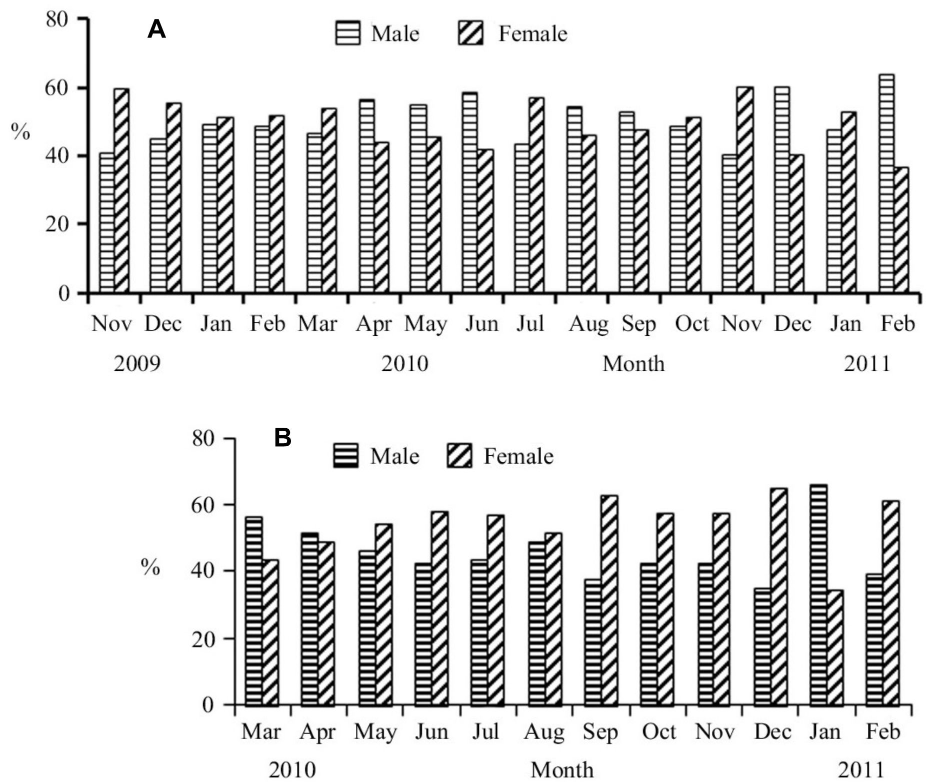

Figure 16 (A) Percentage of males and females *Marcia recens* (Holten, 1802) at Plao Yan between November 2009 and February 2011; (B) Percentage of males and females *Marcia recens* at Chong Lad between March 2010 and February 2011.

3.5.2 Size at first maturity of *Marcia recens* (Holten, 1802)

Size at first maturity is demonstrated the size of short-neck clams that can reproduce at the first time. In a life cycle of short-neck clam, it can reproduce several times. The relationship between the probability of matured male short-neck clams and the shell length (Fig. 17A) was expressed as sigmoid curve. The adjustment of the sigmoid curve in order to find out the relationship equation was carried on by statistical regression analysis. The result of the analysis could provided these values,

$a_{pooled\ male}$ = 2.185, b = -0.112 and the regression coefficient of pool male ($r_{pooled\ male}$) was 0.984

$a_{male\ Plao\ Yan}$ = 2.146, b = -0.121 and the regression coefficient of male ($r_{male\ Plao\ Yan}$) was 0.974

$a_{male\ Chong\ Lad}$ = 4.729, b = -0.195 and the regression coefficient of male ($r_{male\ Chong\ Lad}$) was 0.996

The relationship equations were as following,

$$\text{Probability of mature}_{pooled\ male} = 1/(1 + e^{(2.185 - 0.112\text{Shell length})})$$

$$\text{Probability of mature}_{male\ Plao\ Yan} = 1/(1 + e^{(2.146 - 0.121\text{Shell length})})$$

$$\text{Probability of mature}_{male\ Chong\ Lad} = 1/(1 + e^{(4.729 - 0.195\text{Shell length})})$$

The consideration of fig. 17A found that size at first maturity of male short-neck clam for pooled male was 19.51 mm shell length, for male at Plao Yan was 17.78 mm shell length and male at Chong Lad was 24.22 mm shell length. While the observation on gonadal development stage under microscope found that the minimum shell length of matured male short-neck clams at Plao Yan was 17.60 mm while the minimum shell length of mature male short-neck clams at Chong Lad was 21.45 mm. The size at first maturity of males at Chong Lad was the highest but The size at first maturity of males from both sampling sites under microscope observation were lower than the size at first maturity from the calculation. However, the size at first maturity of males at Chong Lad was still higher than the size at first maturity of the pooled males.

The relationship between the probability of mature female short-neck clams and the shell length (Fig. 17B) was also expressed as sigmoid curve like as males. The adjustment of the sigmoid curve in order to find out the relationship equation was carried on by statistical regression analysis. The result of the analysis could provided these values,

$a_{pooled\ female}$ = 4.214, b = -0.210 and the regression coefficient of pool male ($r_{pooled\ female}$) was 0.971

$a_{female\ Plao\ Yan}$ = 4.174, b = -0.203 and the regression coefficient of male ($r_{female\ Plao\ Yan}$) was 0.995

$a_{female\ Chong\ Lad}$ = 4.771, b = -0.254 and the regression coefficient of female ($r_{female\ Chong\ Lad}$) was 0.910

The relationship equations were as following,

$$\text{Probability of mature}_{pooled\ female} = 1/(1 + e^{(4.214 - 0.210 \text{Shell length})})$$

$$\text{Probability of mature}_{female\ Plao\ Yan} = 1/(1 + e^{(4.174 - 0.203 \text{Shell length})})$$

$$\text{Probability of mature}_{female\ Chong\ Lad} = 1/(1 + e^{(4.174 - 0.203 \text{Shell length})})$$

The consideration of fig. 17B found that size at first maturity of female short-neck clams for the pooled females was 20.09 mm, for females at Plao Yan was 20.58 and for females at Chong Lad was 18.82 mm, while the observation of gonadal development stage under microscope found that the minimum shell length of matured female short-neck clams at Plao Yan was 20.30 mm while the minimum shell length of matured female short-neck clams at Chong Lad was 18.00 mm. The size at first maturity of females at Plao Yan was a little bit higher than at Chong Lad but The size at first maturity of females from both sampling sites under microscope observation were lower than the size at first maturity from the calculation. However, the size at first maturity of females at Plao Yan was still higher than the size at first maturity of the pooled females.

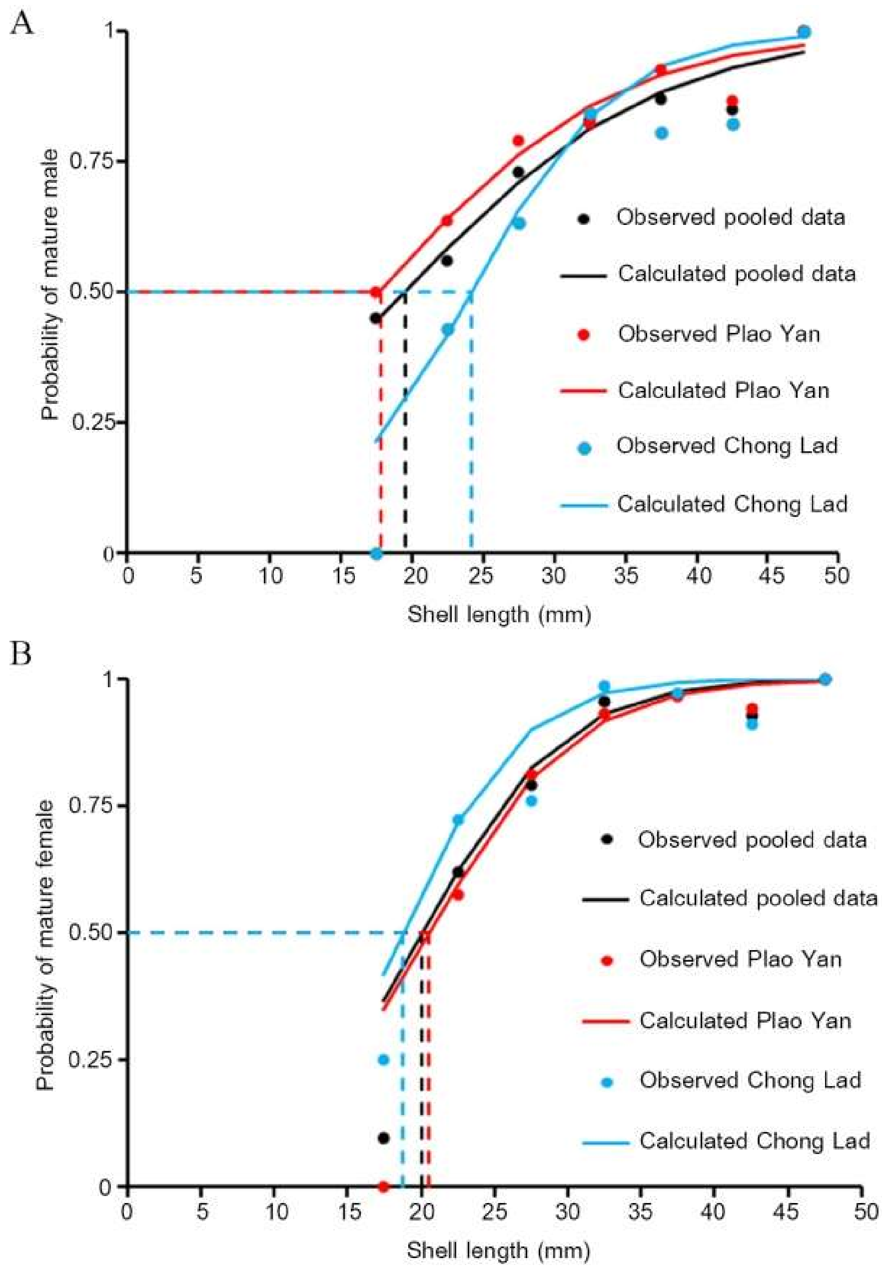

Figure 17 (A) The relationship between probability of mature males *Marcia recens* (Holten, 1802) and the shell length; (B) The relationship between probability of mature females *Marcia recens* (Holten, 1802) and the shell length.

3.6 Gametogenesis and reproductive cycle

3.6.1 Gametogenesis of *Marcia recens* (Holten, 1802)

Histological study of the gonads can distinguish sexes of *M. recens*. It was found that *M. recens* developed their gametes in different stages similar to gametogenesis in other bivalves e.g. cockle, hard clam, short-necked clam and manila clam etc. But the duration of spawning was different for example most of the clams in temperate areas spawned in summer months while the clams in tropical areas spawned throughout the year.

Gametogenesis of *M. recens* in both male and female can be categorized into six stages. Development stages under the examinations of gonad tissues under light microscopy can be described as:

Stage 0, Resting: Gonad predominantly composed of connective tissue with small cells (arrows) surround the follicle wall. Sexes can not be distinguished at this stage (Fig. 18),

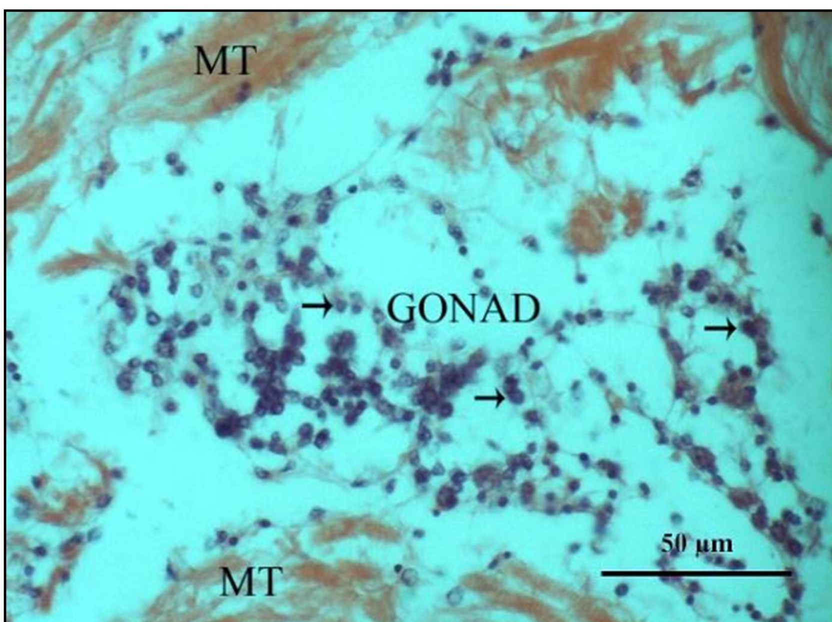

Figure 18 Photomicrograph of *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 0 (Resting); MT= muscular tissue.

Stage 1, Early Development: At this stage, sex can be distinguished and gonad proliferation began. In female gonad, number of perceptible oocytes in follicle walls is increasing; oocytes still small; free oocytes absent in the lumen (Fig. 19). In male gonad, each follicle fill with many follicle cells, spermatogonia attach follicle walls, spermatocytes also present except for spermatids and spermatozoa (Fig. 20).

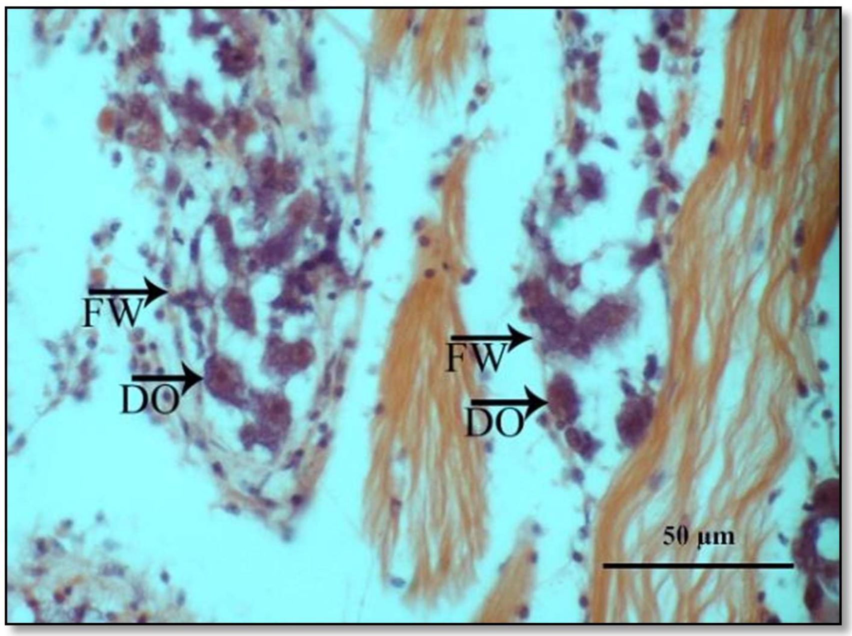

Figure 19 Photomicrograph of female *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 1 (Early development); DO = developing oocyte, FW = follicle walls.

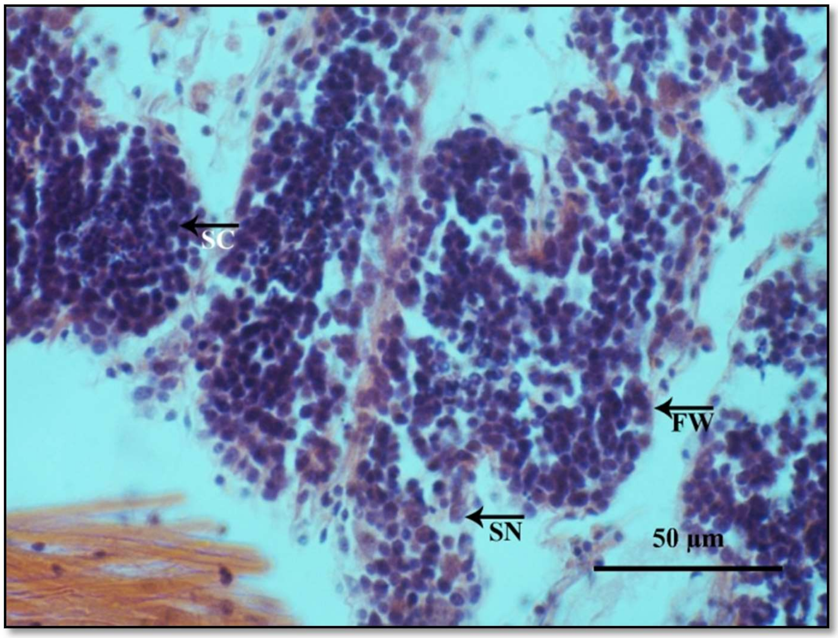

Figure 20 Photomicrograph of male *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 1 (Early Development); FW = follicle wall, SC = spermatocyte; SN = spermatogonia.

Stage 2, Late Development: At this stage, there are free oocytes in the lumen of female gonad but the amount is less than half of all oocytes exist in the follicles; attached oocytes evenly abundant (Fig. 21). In male gonad, spermatogonias, spermatocytes, spermatids and spermatozoan present in follicles; in less developed gonads, dominant cell type cannot be found; in more developed gonads, Most of follicles composed of spermatids and spermatozoa (Fig. 22).

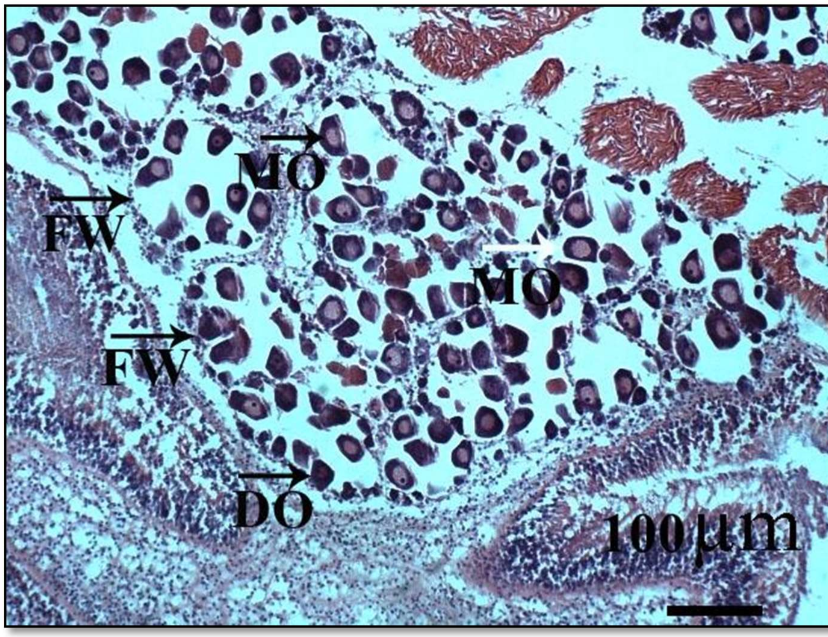

Figure 21 Photomicrograph of female *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 2 (Late Development); DO = developing oocytes, FW = follicle wall; MO = mature oocyte.

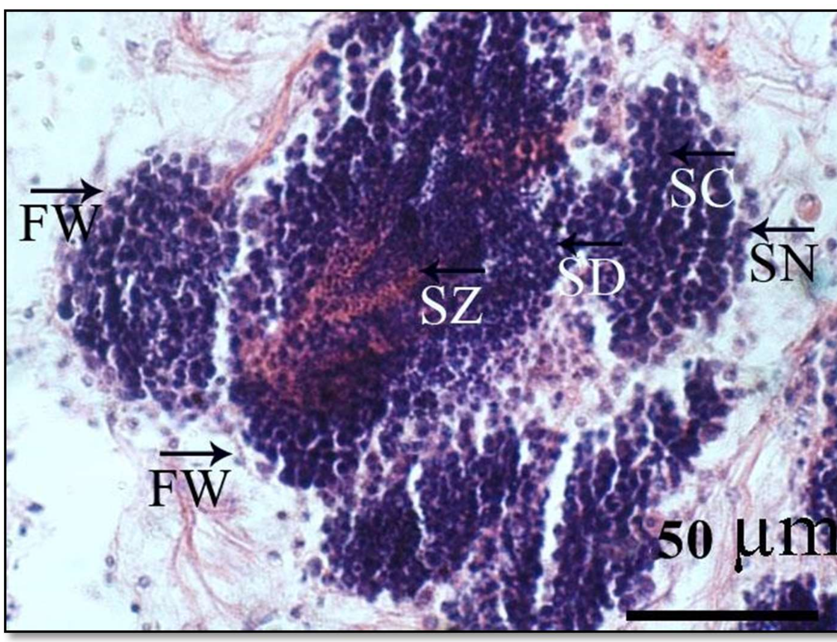

Figure 22 Photomicrograph of male *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 2 (Late Development); FW = follicle wall, SC = spermatocyte, SD = spermatid; SN = spermatogonia; SZ = spermatozoa.

Stage 3 Ripe or mature: At this stage**,** in female, gonad occupy large surface area; most oocytes free in the follicle with a polygonal form, follicle walls thin (Fig. 23). In male follicles mainly composed of mature spermatozoa with their flagellum pointing towards the centre of the follicle forming concentric bands or plugs; spermatozoa bands close to the follicle wall in well-developed ripe specimens; The appearance of follicles is neat and orderly (Fig. 24).

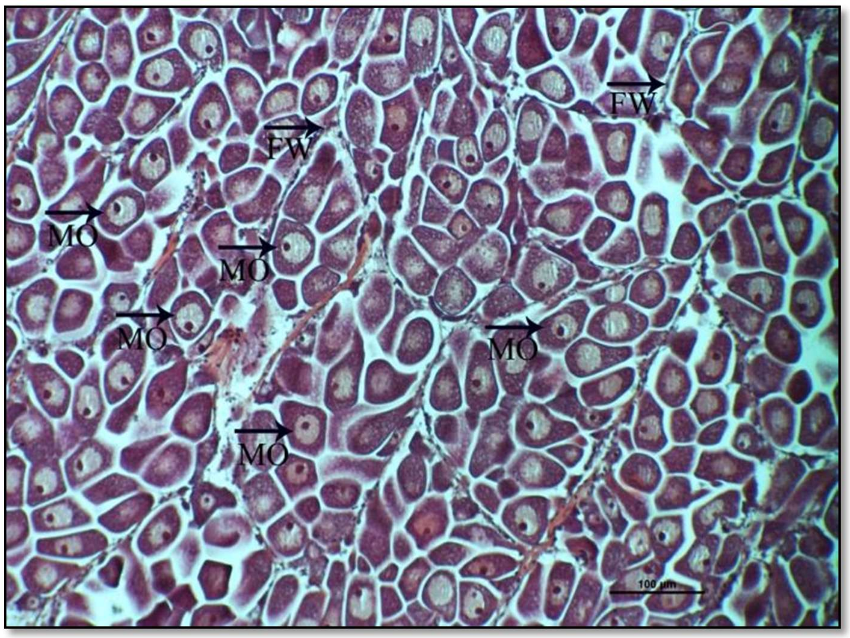

Figure 23 Photomicrograph of female *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 3 (Ripe or Mature); FW = follicle wall; MO = mature oocyte.

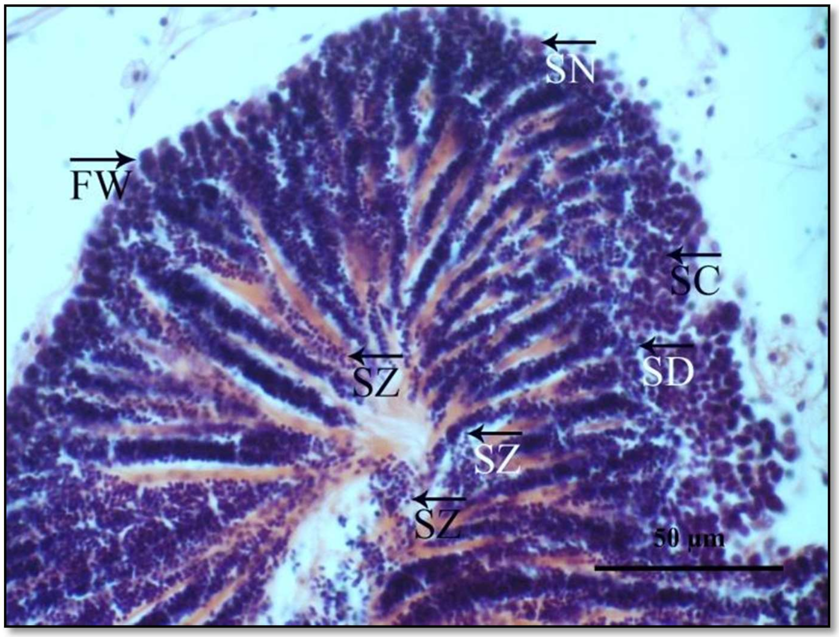

Figure 24 Photomicrograph of male *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province*:* stage 3 (Ripe or Mature); FW = follicle wall, SC = spermatocyte, SD = spermatid; SN = spermatogonia; SZ = spermatozoa.

Stage 4 Partially spent or spawning: At this stage amount of free oocytes in each follicle of female gonad reduced; some follicles empty because of releasing gametes; follicle wall broken (Fig. 25). In male, spermatozoa can be found in a swirling shape and accounting for the main portion of cells in the follicle; empty space in some follicles due to release of mature spermatozoa (Fig. 26).

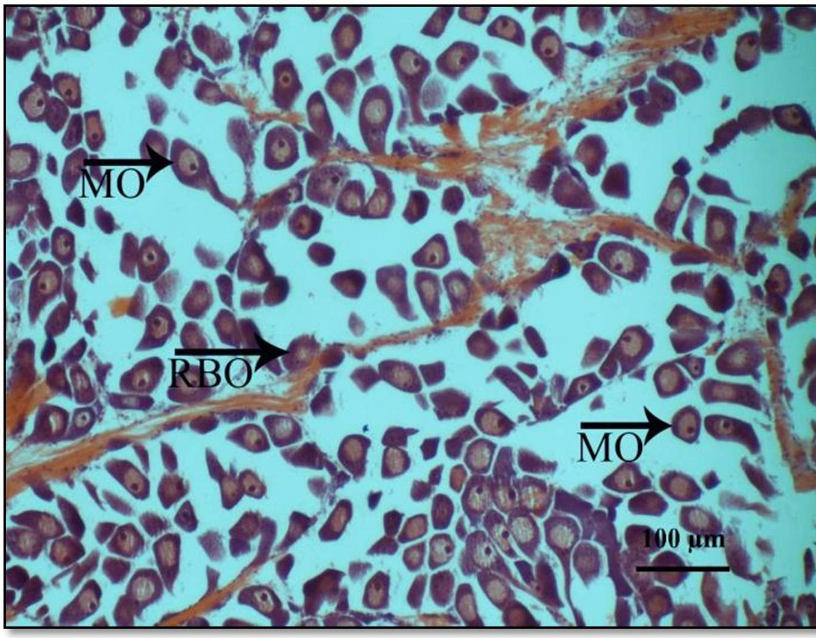

Figure 25 Photomicrograph of female *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 4 (Partially Spent or Spawning); MO = mature oocyte; RBO = resorbing oocyte.

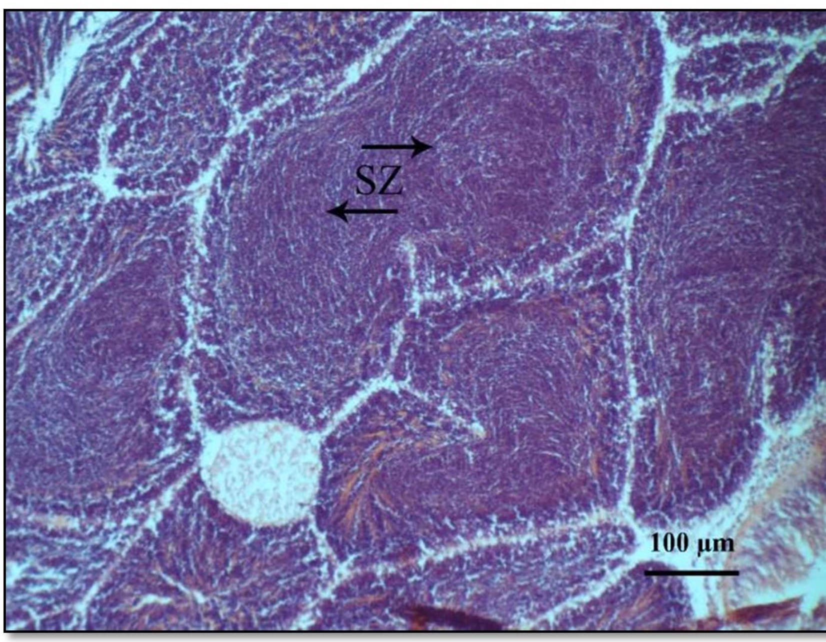

Figure 26 Photomicrograph of male *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 4 (Partially Spent or Spawning); SZ = spermatozoa.

Stage 5 Spent: After spawning, follicle walls were broken, scattered and relatively empty both in male and female; only a few oocytes and spermatozoan remain in female (Fig. 27) and male follicles (Fig. 28), respectively.

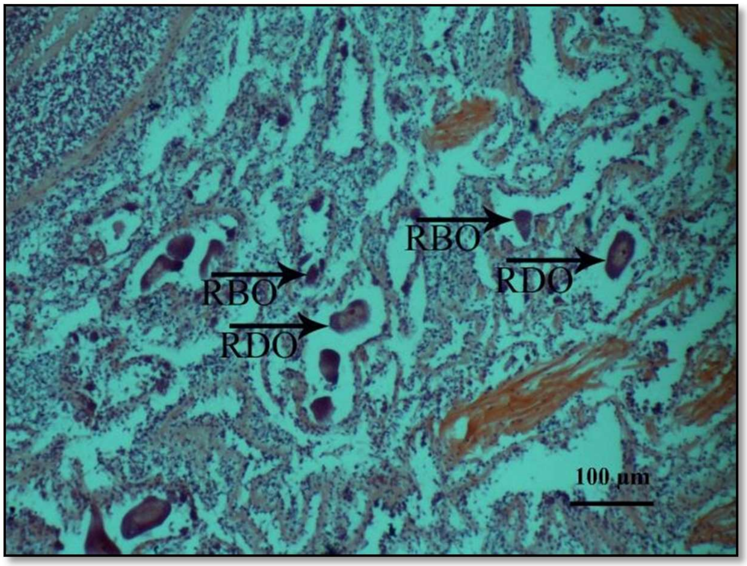

Figure 27 Photomicrograph of female *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 5 (Spent); RBO = resorbing oocyte; RDO = residual oocyte.

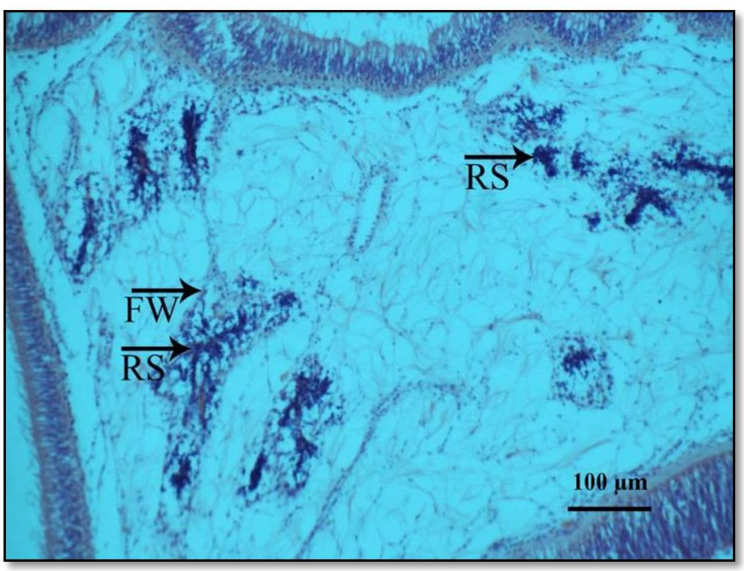

Figure 28 Photomicrograph of male *Marcia recens* (Holten, 1802) gonad (H&E) from Koh Sarai, Satun Province: stage 5 (Spent); FW = follicle wall; RS = residual spermatozoa.

3.6.2 Reproductive cycle of *Marcia recens* (Holten, 1802)

At Plao Yan

Male, the percentage of male clams in various reproductive stages at Plao Yan illustrates in fig. 29A. Gametogenesis was occurred throughout the study period. At the start of the study, in November 2009, half of males were in the ripe stage (Stage 3) and nearly half were in the partially spent stage. The percentage of males in the partially spent stage was continually increased and reached the first peak in January 2010. In February 2010 most of male clams were in the late development stage and the male gonad continually developed as in March 2010 most of male clams were in Ripe stage. In April 2010, the second peak of partially spent stage in males occurred again. At this point, it was found that after the peak spawning of males occurred, the male gonad will take 2-3 months to develop and reach the peak spawning throughout the study period. From the study the first peak of spawning was occurred during December 2009-January 2010, the second peak was occurred in April 2010 and the third peak was occurred in July 2010. Therefore, the peak spawning of male occurred three times in a year.

Female, in November 2009, the beginning of the study, most of female gonads were either in the partially spent stage (Stage 4) or in the ripe stage (Stage 3) (Fig. 29B). The first peak spawning occurred between December 2009 and January 2010. From February until to April 2010, most of females developed their gonad as occurring of Early Development to Ripe stage (Stage 1-3) in higher percentage than Partially spent (Stage 4). The second peak spawning was occurred between May and June 2010, and decreased in the month of July 2010. After that, spawning of females continued from August 2010 until to February 2010. Therefore, the peak spawning of female *Marcia recens* occurred twice a year while the second peak continued for several months.

The statistic test showed that the partially spent stage in males was significantly positive correlated with the ripe stage in females of *M. recens* at Plao Yan (The Pearson product- moment correlation r = 0.498, n = 16, *P* = 0.02). The partially spent stage of female *M. recens* was significantly negative correlated with salinity at Plao Yan (The Pearson product-moment correlation r = -0.543, n = 16, *P* = 0.03). Thus spawning activities increase when salinity becomes low.

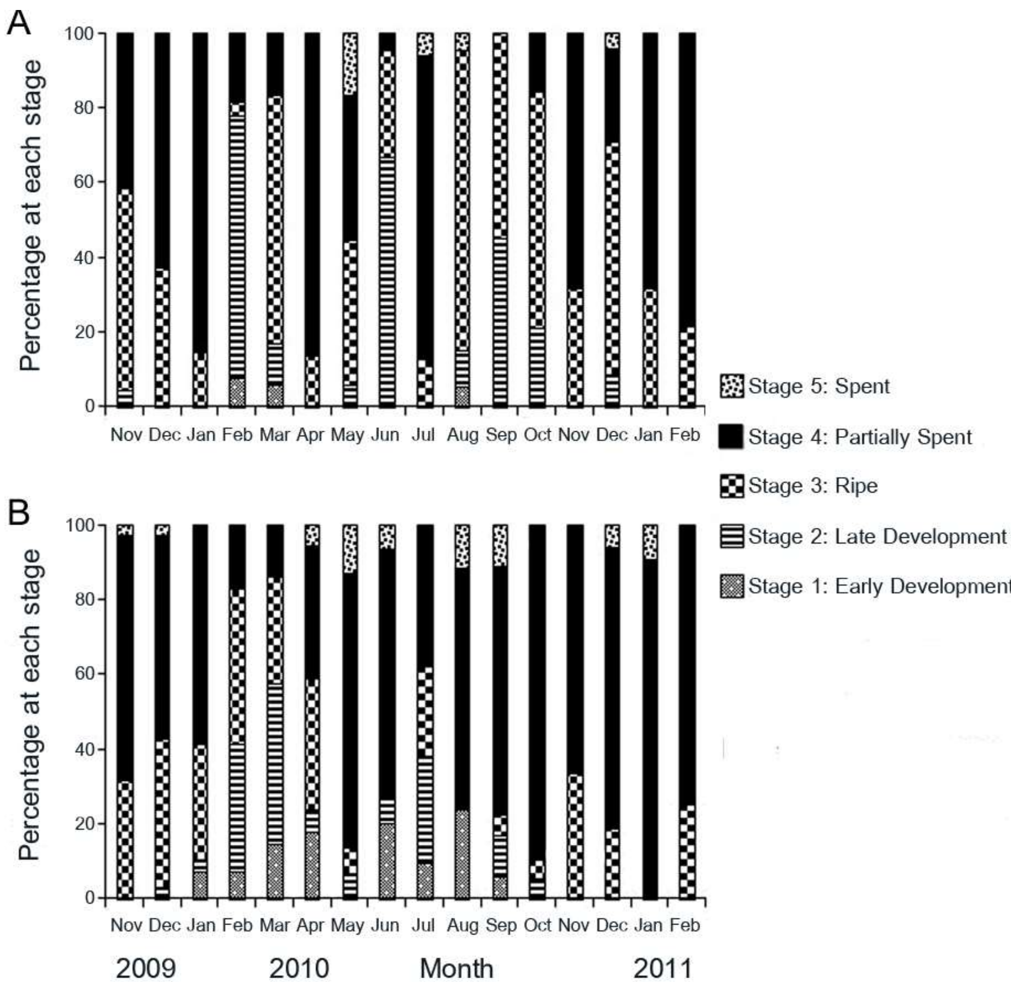

Figure 29 The percentage of various reproductive stages of (A) male and (B) female *Marcia recens* (Holten, 1802) at Plao Yan between November 2009 and February 2011

At Chong Lad

Male. The proportions of male short-neck clams in various reproductive stages at Chong Lad illustrated in fig. 30A. At the beginning of the study, in March 2010, the proportion of late development, ripe and partially spent stage was 36%, 45% and 18%, respectively. Spawning appears continued with three peaks which were in April 2010, July 2010 and January 2011. The majority of clams in these three months was in partially spent and ripe stages, respectively. Between August and October, the majority of sample was made up of late development and ripe specimens, just only a small proportion of spawning (13.33% of partially spent stage found in September 2010). From November the spawning started to increase as evidenced by the presence of partially spent (Stage 4) specimens in the sample and reached the maximum in January 2011 (52% of partially spent stage).

Female. As was the case for the male specimens in these sampling site, there were 3 stages of late development, ripe and partially spent in the percentage of 29, 17 and 52, respectively at the commencement of the study (fig. 30B) and the partially spent specimens were the main in all samples stand out against with the male that ripe specimens were the main in the samples. Spawning of female clams occurred throughout the study period. Since the initiation of the study in March 2010, female short-neck clams developed their gonads in the partially spent stage more than 50% until to June 2010. Spawning decreased in July 2010 as indicated by the low percentage (less than 50%) of the partially spent stage. Subsequently, between August and October 2010 spawning again increased to over 50%. At last, between November 2010 and February 2011, the percentage of spawning (partially spent stage) alternated decreased and increased. Additionally, the gametogenesis was in ripe and partially spent stage in almost every month except for July which was in an early development stage.

The statistic test showed that the ripe stage in male was positive correlated with the ripe stage in female of *Marcia recens* at Chong Lad (The Pearson product-moment $r = 0.546$, $n = 12$, $P = 0.66$) while the environmental conditions of the sea water were not correlated with the gametogenic cycle in both sexes of *Marcia recens*.

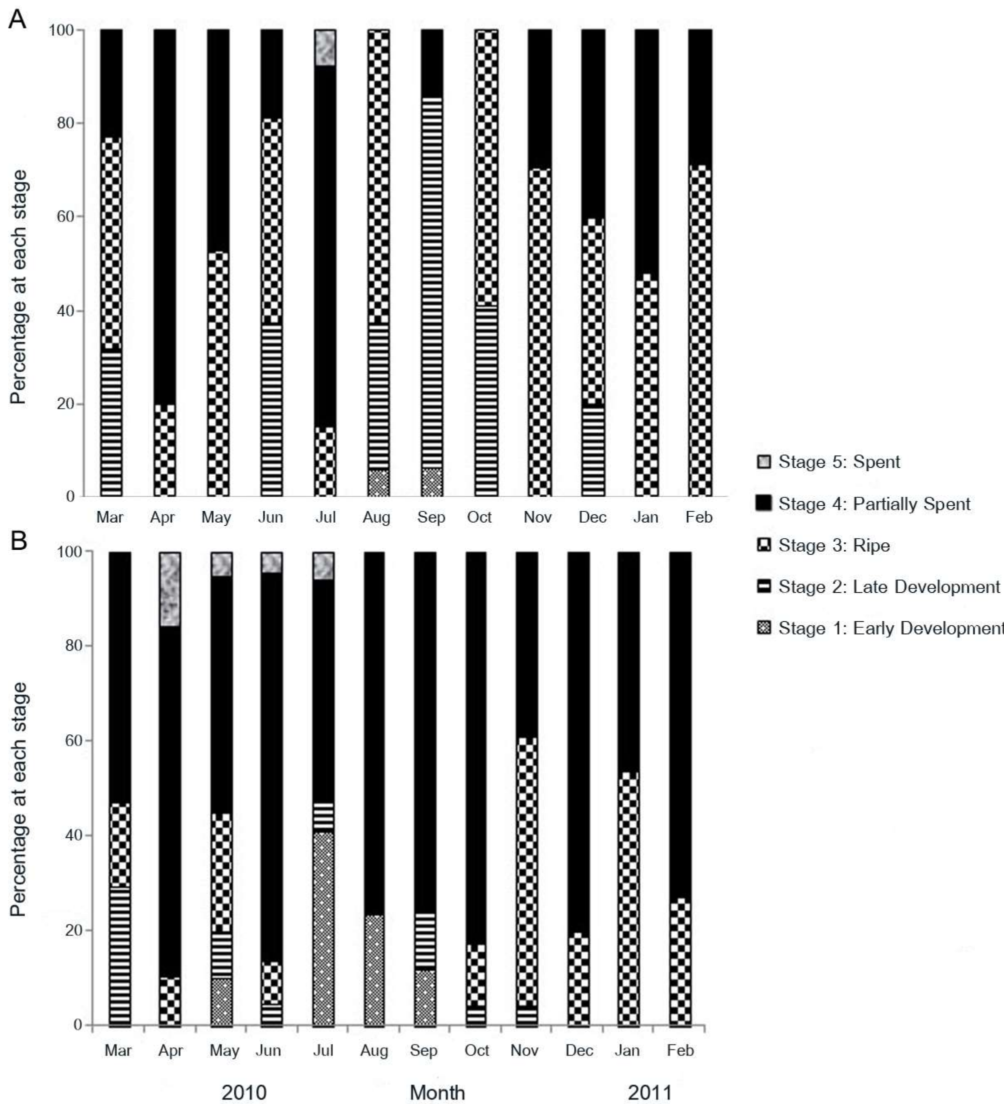

Figure 30 The percentage of various reproductive stages of (A) male and (B) female *Marcia recens* (Holten, 1802) at Chong Lad between March 2010 and February 2011.

3.7 Condition index and gonadal index

3.7.1 Condition Index

The average codition index (CI) of *Marcia recens* at Plao Yan was 9.24 ± 1.97 with its ranged between 7.54 ± 1.38 and 10.67 ± 1.85 (Fig. 31). There was a decrease of the CI between March 2010 and June 2010, and increase between June

2010 and October 2010 and drop down again in November 2010. Indices reached their highest values in February 2011.

The average CI of *Marcia recens* at Chong Lad was 8.85 ± 2.23 with its range between 7.09 ± 1.43 and 12.25 ± 1.87. The lowest value of 7.09 was found in November 2010. A sudden increase of condition index was occurred in the following month, December 2010, reaching the highest condition index of 12.25.

Overall condition indices appear to be lower for clams at Chong Lad than those at Plao Yan site, except for the month of June, September and December 2010 and January 2011.

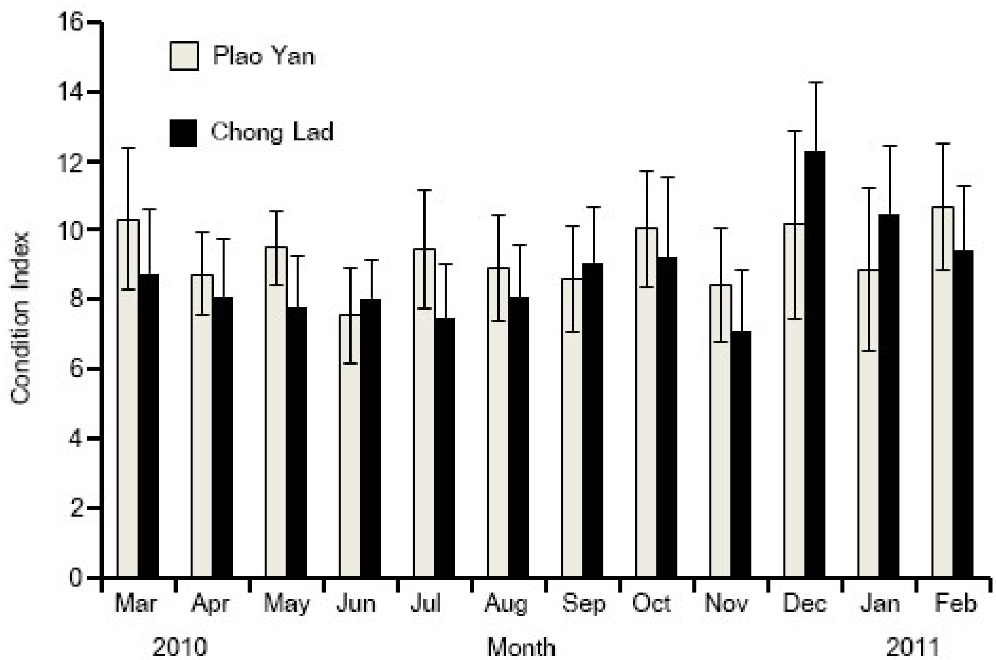

Figure 31 Condition index (CI) of *Marcia recens* (Holten, 1802) at Plao Yan and Chong Lad between March 2010 and February 2011

3.7.2 Gonadal index

Gonadal index, an estimation of the reproductive activity among individuals in a population at Plao Yan ranged between 2.31 and 4.60 for male and slightly lower for female, between 1.90 and 3.86. The gonadal index (GI) of males ranged between 2.31 and 4.60 which the lowest was found in July 2010 and the highest was found in August

2010. While the GI of females ranged between 1.90 and 3.86. The lowest GI was found in January 2011 and the highest GI was found in March 2010 (Fig. 32).

At Chong Lad, the GI of males ranged between 2.38 and 4.59 which the lowest was found in July 2010 and the highest was found in October 2010. The GI of females ranged between 2.05 and 3.78. The lowest GI was found in June 2010 and the highest GI was found in November 2010 (Fig. 32).

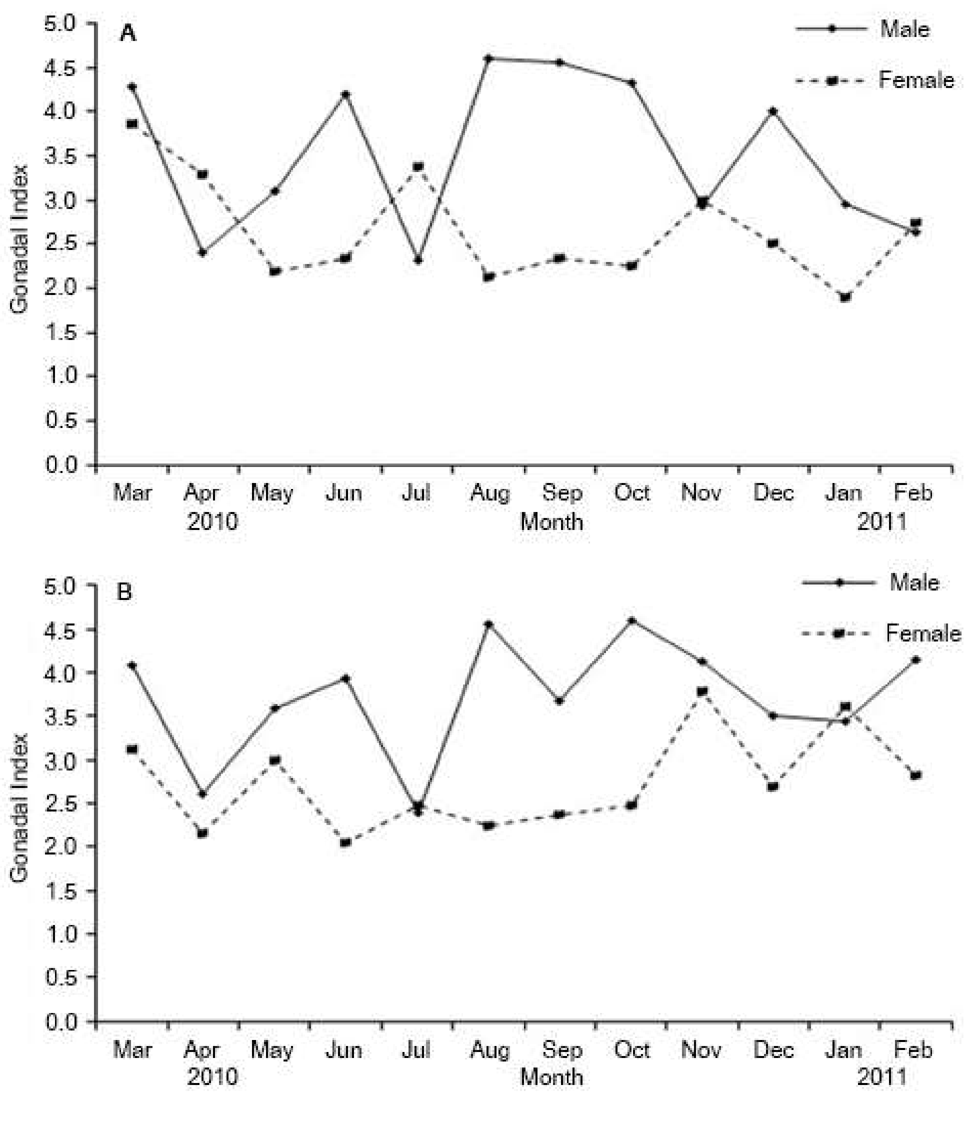

Figure 32 Seasonal variation in gonadal index (GI) of male and female *Marcia recens* (Holten, 1802) at Plao Yan (A) and Chong Lad (B) between March 2010 and February 2011

Chapter 4

Discussion

4.1 Environmental Conditions

Water temperatures ranged from 27-31 ˚C at Plao Yan, and from 26-32 ˚C at Chong Lad (Fig. 5), not varying greatly, which is typical for a tropical environment. It was also similar to the water temperature reported in the habitats of leather Donax clam *Donax scortum* at Pakmeng beach, (Peerakeitkhachorn, 1995) and hard clam *Meretrix casta* in Sikao (Tanyaros and Tongnunui, 2011), Trang Province.

The amount of oxygen dissolved in water (DO) also plays an important role on the survival and distribution of marine organisms. The lowest DO concentration at Plao Yan and Chong Lad was 5.08 mg/l (Fig. 8), which was still higher than the value of less than 4 mg/l recommended by the Pollution Control Department (1994) for the conservation of natural habitats and the reproduction of marine organisms. The pH values (Fig. 7) in this study (Plao Yan: 7.17 - 8.24; Chong Lad: 7.25 - 8.12) were in the 7.5 to 8.4 range for marine habitats (Zeebee and Wolf-gladrow, 2001).

Salinity at both sites fluctuated between 21 and 36 ppt during the sampling periods. High salinity was found in the dry season, with lower values recorded during the rainy season. This was due to the sites being shallow, and near to coastal areas, meaning that the rainfall added large amounts of freshwater to the sea, resulting in a varying of salinity between the seasons.

Chlorophyll a, which is an indicator of phytoplankton biomass and a source of food for bivalve molluscs (Sahin et al., 2006), fluctuated highly in the Koh Sarai area. This variability was probably due to seawater exchange between the open-sea and the Koh Sarai estuary. A similar chlorophyll a condition was found in the habitat of carpet shell clam *Tapes decussatus* in Sufa Lagoon, Turkey (Sedar and Lok, 2009).

Similar soil textures were found in the habitats of *Marcia recens* at Plao Yan (Table 1) and Chong Lad (Table 2). However, the soil organic matter at Plao Yan was higher than at Chong Lad, while the average density of *M. recens* at Plao Yan was lower than at Chong Lad (Fig. 11 and 12). The percentage of sand at both sampling sites was similar to the habitat of Manila clam *Tapes philippinarum* in Korean tidelands (Choon

Koo, 1968). Soil texture and organic matter content may be the most important factors for bivalve life and tends to restrict the distribution of bivalves to different habitats (Choon Koo, 1968). For example, the distribution of *Nuttallia obscurata* was confined to a sand and silt-sand bottom (Kplpakov and Kolpakov, 2005).

Environmental parameters reported in this study fluctuated in a similar way to the study of coastal hydrography of the southern Andaman Sea of Thailand from Trang and Satun Provinces. It reported temperature, dissolved oxygen, pH, and salinity values that ranged from 27.6-29.3 ºC, 5.5-6.4 mg/l, 8.06-8.15, and 32.6-32.8 ppt., respectively (Khokiattiwong, 1991).

The effects of environment conditions on marine organisms have been widely studied, especially those related to water temperature and salinity. In tropical regions, where the variations of water temperature are minimal, even a slight increase in temperature is known to correspond with the period of bivalves breeding (Suja and Muthiah, 2007). Changes of salinity affect a wide variety of biochemical and physiological processes in marine bivalves (Lee et al., 2004). Salinity plays an important role in the biological activities of bivalves, including in their reproductive cycle (Jayabal and kalyani, 1986; cited by Nell and Paterson, 1997). Suja (2002) reported that the maximum growth rate and 100% survival of spat of *M. opima* occurred at a salinity of 25 ppt. In the case of the hard clam *Meretrix meretrix,* temperature is not the main factor for inducing its spawning, but salinity is possibly a factor (Kasoroek, 2000). Changes of temperature and salinity resulting in an increase or decrease of free amino acid (FAA) levels in the tissues of marine bivalves are often monitored as a stress indicator (Lee et al., 2004). However, the temperature and salinity ranges found in this study were similar to those found in the habitats of baby clam *Marcia opima* in Indian waters, namely between 24 to 34 ºC and 26 to 37 ppt, respectively (Suja and Muthiah, 2007).

The breeding intensity of crustaceans and mollusks is also influenced by environmental conditions. For example, the reproductive cycle in this study demonstrated distinctive peaks, with the first occurring during December and January, and the second starting in June. However the second peak was not distinctive, as it carried on for several months. This was due to the environmental conditions gradually changing, as compared to their changes during the first peak. This was similar to the study of baby clam *Marcia opima*, where there were two spawning periods (Varadarajan and Subramoniam, 1982;

cited by Suja and Muthiah, 2007). The peaks occurred during the gradual fall in temperature and salinity in the southwest monsoon season (June to September), and during an increase in temperature and salinity in the summer months and in the post monsoon months respectively (Suja and Muthiah, 2007).

4.2 Sex ratio and Size at First Maturity

4.2.1 Sex ratio of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

The male-to-female sex ratio of the short-neck clam, *Marcia recens,* was 1:1.07 (Table 5), which indicates that *M. recens* is a dioecious organism. This corresponds to other clams in the genus *Marcia*, such as *Marcia opima* (Suja and Muthiah, 2007) and *Marcia hiantina* (Toral-Barza and Gomez, 1986). Some hermaphrodites were also encountered in this study but in a low percentage. In general, the ratio of the sexes for dioecious bivalves is approximately 1:1, with the number of females often being slightly higher than the number of males (Gosling, 2003; Nabuab and del Norte-Campos, 2006). Previous studies on bivalve reproduction have found that the sex ratio was 1:1, including for *Gafrarium tumidum* (Jagadis and Rajagopal, 2007), *Donax trunculus* (Gaspar et al., 1999; Silina, 2006), short-necked clams *Paphia undulata* (Jindalikit, 2000), *Anadara granosa* (Thongchai, 2008), Blood cockles *Anadara inaequivalvis* (Sahin et al., 2006), *Scrobicularia plana* (Rodriguez-Rua et al., 2003), and the Noah's ark shell *Arca noae* (Peharda et al., 2006).

Thongchai (2008) and Bantoto and Ilano (2012) stated that if the number of male and female bivalves is in equilibrium, then the eggs released from each fecund female will have a higher chance of being fertilized by males, and reproductive success is assured. However, a dominant sex was found in some other bivalve species. For example, male dominance was reported for the manila clams *Ruditapes philippinarum* (Drummond et al., 2006) and the leather Donax clam *Donax scortum*. (Peerakeitkhachorn, 1995). Female dominance was reported for the carpet shell clams *Tapes decussatus* from Sufa lagoon, Turkey (Serdar and Lok, 2009) and for cockle *Anadara granosa* (Suwanjarat and Parnrong, 1991). Also, the sex ratio of baby clams *Marcia opima* from the southeast coast of India changed toward a female bias, while those from the southwest coast changed toward a male bias (Suja and Muthiah, 2007). The sex bias phenomenon can be observed in nature based on environmental conditions. Kandeel et al. (2013) explained that the sex bias for the cockle *Cerastoderma glaucum* in Lake Qaran, Egypt occurred because of three possibilities: (1) the presence of sexually undifferentiated individuals at a completed spawning stage, (2) as a reflection of differences in the developmental tempo of the two sexes, and (3) that males or females are more sensitive to unfavorable environmental conditions. For instance, the sex ratio in pearl oyster *Pinctada magaritifera* changed toward a male bias when there was low food availability (Chavez-Villalba et al., 2011).

4.2.2 Size at first maturity of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

The average shell length at first maturity of male and female *Marcia recens* were 19.51 and 20.09 mm (Fig. 17) respectively, which are relatively small compared to previous studies by Tan and Sigurdssan (1984) in Singapore waters, which reported a shell length of 25.00 mm. However, our finding is still inside the ranges for other bivalves in the family Veneridae, which vary between 10 and 50 mm in shell length. The smallest size at first maturity (10 mm) was found in *Ruditapes decussatus* (Lucas, 1968; cited by Gosling, 2003). Shell lengths between 17 and 20 mm were found in hard clam *Meretrix lusoria* in Japanese waters (Nakamura et al., 2010), manila clam *Ruditapes philippinarum* (Dang et al., 2010; Chung et al., 2013), *Chamelea gallina* (Dalgic et al., 2009), and *Gafrarium tumidum* (Baron, 1992; Jagadis and Rajagopal, 2007). Shell lengths of between 40 and 50 mm were reported for hard clam *Meretrix petechialis* (Jun et al., 2012), short-neck clam *Paphia undulata* in Philippine waters (Nabuab et al., 2010), and *Megapitaria squalida* in the southeast Gulf of California, Mexico (Villalejo-fuerte et al., 2000; cited by Alvarez Dagnino et al., 2017).

4.3 Gametogenesis and Reproductive Cycle of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

4.3.1 Gametogenesis of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

There were 2-4 different reproductive stages in each follicle of both the male and female *M. recens* at the same time. These include a ripe and partially spent stage, an early development to partially spent stage, and a late development to spent stage in the follicles. *M. recens* released gametes several times in each cycle of their gametogenesis, which suggested that they employ continuous reproduction. The short-neck clam *Paphia undulata* from Samut Sakhon were similar in that the matured-gametes spawned while the rest were in the process of maturing (Jindalikit, 2000). This is the typical pattern of reproduction in tropical species, which adopt opportunistic strategies to develop their gonads from food that is available all year round rather than from energy stored within somatic tissues (Freites et al., 2010). Many animals cannot be defined definitively by one reproductive stage (Suja and Muthiah, 2007). For example, in an individual clam, many follicles undergo different reproductive stages at the same time, as in the tropical blacklip pearl oyster *Pinctada margaritifera* (Pouvreau et al., 2000); the manila clam *Tapes phillipiinarum* (Drummond et al., 2006); the eared ark *Anadara notabilis* (Freites et al., 2010); and the blood cockle *Anadara antiquata* (Afiati, 2007).

4.3.2 Reproductive Cycle of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

This study employed five stages of reproductive maturity, plus a sexually undifferentiated stage, for male and female short-neck clams. This approach has been utilized by most authors for venus clam reproductive biology (Suwanjarat and Parnrong, 1991; Drummond et al., 2006; Suja and Muthiah, 2007 and Jagadis and Rajagopal, 2007).

The results are similar to those from previous studies of the gametogenic cycle of the bivalves in tropical waters, confirming that spawning occurs continuously throughout the year. For instance, Afiati (2007) mentioned that spawning in both *Anadara granosa* and *A. antiquata* of Central Java progresses gradually throughout the year, as indicated by the presence of various stages of oogonia and spermatogonia. Similar results were reported for *Gafrarium tumidum* from the southeast coast of India (Jagadis and Rajagopal, 2007). The spent stage was rarely found in this study, implying that gonad development of *M. recens* is persistent throughout the year, which was similar to the gonad development of the elongated sunset clam *Gari elongata* in the West Central Phlippines (Nabuab and Norte-Campos, 2006). In tropical regions, where the variations in water temperature are minimal, even a slight increase in temperature corresponds to periods of breeding (Suja and Muthiah, 2007; Kastoro, 1995).

In Tambon Koh Sarai, Satun Province, the high percentage of the partially spent (spawned) stage, and the very low percentage or absence of the spent stage in the gonad of *M. recens*, indicates that gonad development is rapid. This agrees with the investigation by Suwanjarat et al. (2009) which stated that the gametogenesis of the clam *Anadara granosa* from Pattani Bay occurs rapidly; the gametes are released as soon as they mature, and the recovery is fast. In a similar way, *Argopecten purpuratus* has the ability to replace gametes within about 20 days of spawning (Cantillanez et al., 2005). The rapid gonad recovery periods in bivalves may be due to the suitablity of the environment conditions, especially water temperature. For example, Chavez-Villalba et al. (2002), cited by Chavez-Villalba et al. (2007), observed that *Crassostrea gigas* in early gametogenesis can reach reproductive maturity after 7 days under an optimal temperature (22 ˚C). The rapid gonad recovery periods in bivalves may be due to the availability of food (Kastoro, 1995). For example, Enriquez-Diaz et al. (2009) discovered high proportions of mature oocytes in pacific giant oysters *Crassostrea gigas* located in zones with accessibility to better food sources. This agrees with this study, in which the ripe stage and the partially spent stage showed higher proportions than other stages.

Macia recens is a dioecious organism, with small numbers of hermaphrodites (0.25%). Nabuab et al. (2010) found a small percentage of hermaphrodite short necked clam *Paphia undulata* in Central Philippines, but that species is still functionally dioecious. Drummond et al. (2006) mentioned that the occurrence of hermaphrodites in a dioecious mollusc illustrates that this is an unusual incidence. The unnatural condition of the environment may have caused hermaphroditism of the clams. Suja and Muthiah (2007) did not observe any hermaphrodites in a sample of 1,020 baby clams *Marcia opima*, confirming that the occurrence of hermaphrodites is a rare event for this genera. In Thailand, the sex change of clams has not been reported in blood cockles *Anadara granosa* (Suwanjarat and Parnrong, 1991, Suwanjarat et al., 2009), Leather Donax *Donax scortum* (Peerakeitkhachorn, 1995), or short necked clams *Paphia undulata* (Jindalikit, 2000).

4.4 Condition Index and Gonadal Index of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

4.4.1 The Condition Index (CI) of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

The condition index (CI) of *M. recens* (Fig. 31) does not explain gonadal development, which increases during spawning and decreases during gametogenesis. Drummond et al. (2006) stated that the maximum CI corresponds to a peak in ripeness of clams following an accumulation of resources in preparation for spawning. Chavez-Villalba et al. (2007) found that there was no relationship between the condition index and reproduction in oyster *Crassostrea gigas*, which is similar to this study because the highest condition index values occurred at the same time as the high percentage of the partially spent stage. Lodeiros and Himmelman (1999) found that the main factor that influenced gonadal growth in bivalves is the chlorophyll concentration, indicating that gamete production is stimulated by the increase in phytoplankton. Barraza-Guardada et al. (2009) used CI to measure the nutritive state of oysters, and found that high levels of chlorophyll *a* was detected during summer, but the highest CI occurred in winter. The result of this study is similar in that the lowest levels of chlorophyll a at Chong Lad was detected in December 2010, but the maximum CI also occurred in the same month. Choi and Chang (2003) stated that the CI cannot be a reliable direct indicator of the spawning season of bivalves. The results from this study confirm that CI does not correlate with the reproductive cycle of *M. recens*, which is similar to the result of Herrmann et al. (2009) who found that the CI was not useful as it did not significantly correlate with the gametogenic cycle of *Mactra mactroides*. However, a relationship between the condition index and gametogenic activity of clam has been observed in several species inhabiting Thai waters, such as *Anadara granosa* (Thongchai, 2008), *Donax scortum* (Peerakietkhachorn, 1995), *Meretrix* sp. (Tuaycharoen and Prompai, 1991).

4.4.2 The Gonadal Index, GI of *Marcia recens* (Holten, 1802) in Tambon Koh Sarai

The mean GI values of male and female *M. recens* ranged from 2.31-4.60 and 1.90-4.00 respectively (Fig. 32) suggesting that most of the clams were undergoing maturation; a similar condition was also found in *Lutraria philippinarum* in the Philippines

(Bantoto and Ilano, 2012). The GI of *M. recens* in this study confirmed the results of Suwanjarat et al. (2009) that the GI of *Anadara granosa* in Pattani Bay follow the same pattern as gonad development. Generally, the GI values in bivalves increase during gametogenesis and decrease during spawning and resting, in a similar way to *M. squalida* (Adjei-Boateng and Wilson, 2013). The higher decrease in the weight of the clam, reflected by the gonadal index, shows the lowest value after spawning (Alvarez-Dagnino et al., 2017). Mladineo et al. (2007) stated that the gonadal index of *Mytilus barbatus* had a significant positive correlation with sea surface temperature while there was a negative correlation with salinity. However, *Arca noae* present in the same habitat as *M. barbatus*, showed no statistical correlation between temperature and gonad index (Peharda et al., 2006). The GI of *M. recens* in this study was also not correlated with any of the environmental parameters.

CHAPTER 5

Conclusions

The reproductive biology of *Marcia recens* (Holten, 1802) at Tambon Koh Sarai, Satun Province, southern Thailand were examined with particular emphasis on their gametogenesis and reproductive cycle. *Macia recens* is dioecious with a sex ratio close to 1. Male and female *M. recens* at first maturity reach about the same size of approximately 2.0 cm in shell length. Their reproductive cycles appear to have two spawning peaks, which are similar for males and females, except for minor differences between the longevity of the spawning period which lasted throughout the study period. The high percentage of partially spent activities for the females stretch over the middle of rainy season and the middle of hot season, while the high percentage of partially spent activities for the males occurred every 1 - 2 months.

Turning to the question, “How temporal and spatial differences affect reproductive characteristics of *M. recens* at Tambon Koh Sarai?” There was no significant difference between the Plao Yan and Chong Lad sampling sites for *M. recens* reproductive characteristics such as size at first maturity, sex ratio, condition index, and gonadal index. However, throughout the study period, some reproductive characteristics changed in each sampling, including sex ratio, condition index, and gonadal index. *Macia recens* from Tambon Koh Sarai, Satun Province, southern Thailand, displayed continuous recruitment with a suggested seasonal pattern of reproductive activities.

Environmental factors such as temperature, salinity, pH, Chlorophyll *a*, and organic matter in sediment show no correlation to *M. recens*’ reproductive cycles at Chong Lad. However, female clams at the Plao Yan sampling site did display a negative correlation with salinity, which meant that the spawning activities of female *M. recens* increased while salinity decreased.

RECOMMENDATIONS

Short-neck clams *M. recens* are harvested from natural beds for the market in a similar way to other bivalves. Therefore, proper management strategies, rules, regulations, and laws, are essential to ensure their sustainable production. For example, their harvested size must be larger than the size at first maturity, i.e. more than 2.1 cm in shell length. This will require harvested zoning to allow the population to recover without continuous disturbance. Further studies should be carried out on various aspects, such as: the habitats for very small size clams (which were not found in this study), harvested size, and time for a population to recover. Monitoring after the implementation of a management plan is essential to assess if the plan needs adjustment.

In general, stakeholders must be aware of the importance of their resources as well as having better tools for management. Management success depends on the quality of the people, and capacity building for stakeholders is very important. Understanding the biology and ecology of the organisms is essential.

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APPENDICES

APPENDIX A

Fixatives solution preparation

1. Neutral buffered formalin (pH 7.0) 1 litre

(37-40% formaldehyde) formalin	100.00 ml
Disodium hydrogen phosphate anhydrous (Na_2HPO_4)	6.50 g
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$)	4.00 g
Distilled water	900.00 ml

Neutral buffered formalin, usually simply shortened to NBF, has become the standard fixative for use in a diagnostic setting. It is more effective than the simple formalin mixtures as the phosphate salts present make it unlikely that erythrocytes will be damaged, and the neutral pH inhabits the formation of formalin pigment. The phosphate will adjust the pH to about 7.0 as the "neutral" infers, but there is no need to adjust it to this level if it is slightly different.

This fixative should be applied overnight as a minimum, but fixation is not complete until applied for a few days, and a week or two is not too long. For thorough fixation the proteins in the tissue need to be crosslinked, but it is well known that simple formalin mixtures discolor tissues well before they crosslink the proteins. For that reason visual observation does not give any indication as to the degree of fixation, and discoloration should never be used as an indicator that it is complete.

After treatment; NBF contains salts which have limited solubility in high concentrations of ethanol. For that reason the tissues should be transferred to a dehydrant containing 60% ethanol, or less, for a short time in order to give the salts an opportunity to be removed. If transferred directly to 95% or absolute ethanol the phosphate will most likely precipitate on and in the tissue, causing difficulties in sectioning, such as tearing and scoring. Processing machines should be flushed periodically with water to remove accumulated salts.

2 Hematoxylin staining color

Hydrated potassium aluminium sulate ($KAl(SO_4)_2 \cdot 12H_2O$)	50.00 g
Haematoxylin crystal	2.00 g
Sodium iodate ($NaIO_3$)	0.40 g
Citric acid ($C_6H_80_7 \cdot H_2O$)	2.00 g
Chloral hydrate ($CCl_3CH(OH)_2$)	100.00 g
Distilled water	

Put hydrated potassium aluminium sulate 50.00 g into distilled water, stir until soluted. Put sodium iodate 0.40 g, citric acid 2.00 g and chloral hydrate 100.00 g into the solution. Add more distilled water to adjust the volume to be 2,000 ml and mixed well. Keep the solution under the room temperature for a week before using.

3. Eosin staining color

1 gram of Eosin (Y.CI 45380) add in 1 litre of 70% ethyl alcohol and lastly put 5 millilitres of acitic acid. Mixed the solution well and keep in the dark brown bottle under the room temperature.

APPENDIX B

The Gonad tissue processing and embedding according

1. Dehydration: The tissues were put in 70% alcohol, 95% alcohol and absolute alcohol for two hours, twice, respectively and then were placed in isopropyl alcohol for two hours, twice. This step was used 14 hours in total.
2. Clearing: Bring the tissues in no.1 put in Xylene I for an hour.
3. Infiltration: Bring the tissues in no.2 put in Xylene II for an hour
4. Embedding: fix the tissues in paraplast.
5. Sectioning: Cut the wax blocks at 5 µm thickness using the rotary microtome.

APPENDIX C

Staining of tissues

Staining Harris's haematoxylin and counter-stained in eosin, as follows:

1. Dewax in xylene for 2 minutes, twice.
2. Hydrate in absolute ethyl alcohol for 2 minutes, twice.
3. Hydrate in 95% ethyl alcohol for 2 minutes, twice.
4. Wash well in running tap water for 5 minutes.
5. Stain in Harris's haematoxylin for 6 minutes.
6. Differentiate in 1percent acid alcohol for 5 seconds.
7. Wash well in tap water for 2 minutes.
8. Blue by dipping in saturated lithium carbonate for 30 seconds.
9. Wash in distilled water for 1-2 minutes.
10. Stain in eosin for 30 seconds to 1 minute.
11. Dehydrate in 95% ethyl alcohol, twice, for 5-10 dips each.
12. Dehydrate in absolute ethyl alcohol for 2 minutes, twice.
13. Clear in xylene for 2 minutes, twice.
14. Mount with permount.

APPENDIX D

Chi-square test

Table 1 The chi-square test of number of *Marcia recens* (Holten, 1802) at Plao Yan in each month between November 2009 and February 2011.

Month and Year	O_i (M)	O_i (F)	Total	E_i ($n\rho$)	$\frac{(O_i - E_i)^2}{E_i(M)}$	$\frac{(O_i - E_i)^2}{E_i(F)}$	χ^2	Sex ratio M:F
Nov 2009	24	35	59	29.5	1.025	1.025	2.051	1:1.46
Dec 2009	27	33	60	30	0.300	0.300	0.600	1:1.22
Jan 2010	28	29	57	28.5	0.009	0.009	0.018	1:1.04
Feb 2010	27	29	56	28	0.036	0.036	0.071	1:1.07
Mar 2010	18	21	39	19.5	0.115	0.115	0.231	1:1.17
Apr 2010	22	17	39	19.5	0.321	0.321	0.641	1:0.77
May 2010	17	16	33	16.5	0.015	0.015	0.030	1:0.94
Jun 2010	21	14	35	17.5	0.700	0.700	1.400	1:0.67
Jul 2010	16	21	37	18.5	0.338	0.338	0.676	1:1.31
Aug 2010	20	17	37	18.5	0.122	0.122	0.243	1:0.85
Sep 2010	20	18	38	19	0.053	0.053	0.105	1:0.90
Oct 2010	19	20	39	19.5	0.013	0.013	0.026	1:1.05
Nov 2010	17	23	40	20	0.450	0.450	0.900	1:1.35
Dec 2010	24	16	40	20	0.800	0.800	1.600	1:0.67
Jan 2011	19	21	40	20	0.050	0.050	0.100	1:1.10
Feb 2011	14	7	21	10.5	1.167	0.167	2.333	1:0.50
Total	333	337	670	335	0.012	0.012	0.024	1:1.01

M = Male, F = Female

χ^2 table = 3.841, df = 2-1 = 1 (P <0.05)

Table 2 The chi-square test of number of *Marcia recens* (Holten, 1802) at Chong Lad in each month between March 2010 and February 2011.

Month and Year	O_i (M)	O_i (F)	Total	E_i ($n\rho$)	$\frac{(O_i - E_i)}{E_i(M)}$	$\frac{(O_i - E_i)}{E_i(F)}$	χ^2	Sex ratio M:F
Mar 2010	22	17	39	19.5	0.321	0.321	0.641	1:0.77
Apr 2010	20	19	39	19.5	0.013	0.013	0.026	1:0.95
May 2010	17	20	37	18.5	0.122	0.122	0.243	1:1.18
Jun 2010	16	22	38	19	0.474	0.474	0.947	1:1.38
Jul 2010	13	17	30	15	0.267	0.267	0.533	1:1.31
Aug 2010	16	17	33	16.5	0.015	0.015	0.030	1:1.06
Sep 2010	15	25	40	20	1.250	1.250	2.500	1:1.67
Oct 2010	17	23	40	20	0.450	0.450	0.900	1:1.35
Nov 2010	17	23	40	20	0.450	0.450	0.900	1:1.35
Dec 2010	15	25	40	20	1.250	1.250	2.500	1:1.67
Jan 2011	25	13	38	19	1.895	1.895	3.789	1:0.52
Feb 2011	14	22	36	18	0.889	0.889	1.778	1:1.57
Total	207	243	450	225	1.440	1.440	2.880	1:1.17

M = Male, F = Female

χ^2 table = 3.841, df = 2-1 = 1 (P <0.05)

Table 3 Number and percentage of male and female *Marcia recens* (Holten, 1802) at Plao Yan and Chong Lad between November 2009 to February 2011.

Month	Plao-Yan					Chong-Lad				
	male		Female		M:F	Male		Female		M:F
	N	%	N	%		N	%	N	%	
2009										
Nov	24	40.68	35	59.32	1:1.46					
Dec	27	45.00	33	55.00	1:1.22					
2010										
Jan	28	49.12	29	50.88	1:1.04					
Feb	27	48.21	29	51.79	1:1.07					
Mar	18	46.15	21	53.85	1:1.17	22	56.41	17	43.59	1:0.77
Apr	22	56.41	17	43.59	1:0.77	20	51.28	19	48.72	1:0.95
May	17	51.52	16	48.48	1:0.94	17	45.95	20	54.05	1:1.18
Jun	21	60.00	14	40.00	1:0.67	16	42.11	22	57.89	1:1.38
Jul	16	43.24	21	56.76	1:1.31	13	43.33	17	56.67	1:1.31
Aug	20	54.05	17	45.95	1:0.85	16	48.48	17	51.52	1:1.06
Sep	20	52.63	18	47.37	1:0.90	15	37.50	25	62.50	1:1.67
Oct	19	48.72	20	51.28	1:1.05	17	42.50	23	57.50	1:1.35
Nov	17	42.50	23	57.50	1:1.35	17	42.50	23	57.50	1:1.35
Dec	24	60.00	16	40.00	1:0.67	15	37.50	25	62.50	1:1.67
2011										
Jan	19	47.50	21	52.50	1:1.10	25	65.79	13	34.21	1:0.52
Feb	14	66.67	7	33.33	1:0.50	14	38.89	22	61.11	1:1.57
Total	333	49.70	337	50.30	1:1.01	207	46.00	243	54.00	1:1.17

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