REPRODUCTIVE AND DEVELOPMENTAL BIOLOGY OF RICE FIELD CRAB *Esanthelphusa nani* (Naiyanetr, 1984)

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ชีววิทยาการสืบพันธุ์และการเจริญของปูนา *Esanthelphusa nani* (Naiyanetr, 1984)

นายรชตะ มณีอินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	REPRODUCTIVE AND DEVELOPMENTAL BIOLOGY OF RICE FIELD CRAB *Esanthelphusa nani* (Naiyanetr, 1984)
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Field of Study	Zoology
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รชตะ มณีอินทร์ : ชีววิทยาการสืบพันธุ์และการเจริญของปูนา *Esanthelphusa nani* (Naiyanetr, 1984). (REPRODUCTIVE AND DEVELOPMENTAL BIOLOGY OF RICE FIELD CRAB *Esanthelphusa nani* (Naiyanetr, 1984)) อ.ที่ปรึกษาหลัก : ผศ. ดร.นพดล กิตนะ, อ.ที่ปรึกษาร่วม : อ. ดร.จิรารัช กิตนะ,รศ. ดร.จูเลียง โคลดเดอะ

ปูนาเป็นปูน้ำจืดที่มีความสำคัญทั้งในบทบาทการเป็นศัตรูทำลายข้าวและการเป็นแหล่งอาหารสำหรับคนท้องถิ่น บทบาทเหล่านี้ต้องใช้ความรู้ทางชีววิทยาการสืบพันธุ์เพื่อประโยชน์ในการจัดการประชากรในธรรมชาติ งานวิจัยนี้ได้ศึกษาการเปลี่ยนแปลงของระบบสืบพันธุ์และต่อมไร้ท่อของปูนาเพศผู้และเพศเมียตลอดจนการเจริญของเอ็มบริโอ โดยเก็บตัวอย่างปูนาน่าน *Esanthelphusa nani* ตัวเต็มวัยจากพื้นที่จังหวัดน่านในช่วงเดือนพฤศจิกายน 2558 ถึง พฤศจิกายน 2559 นำมาการุณยฆาตและผ่าตัดอวัยวะสืบพันธุ์ ตลอดจนเก็บอวัยวะใกล้กล้ามเนื้อ mandible สำหรับศึกษาต่อมไร้ท่อในเพศผู้ และเก็บ hemolymph เพื่อตรวจสอบการเปลี่ยนแปลง vitellogenin ในเพศเมีย แล้วนำข้อมูลน้ำหนักอวัยวะสืบพันธุ์กับน้ำหนักตัวมาคำนวณค่า gonadosomatic index (GSI)

ผลการศึกษาพบว่า ปูเพศผู้มีค่า gonadosomatic index (GSI) เปลี่ยนแปลงไปตามเวลาโดยมีค่าสูงสุดในช่วงฤดูฝน (สิงหาคม) โดยสามารถแบ่งการเจริญของอัณฑะได้เป็น 3 ระยะ คือ 1) resting stage 2) developing stage และ 3) active stage เมื่อตรวจสอบภายใต้กล้องจุลทรรศน์พบว่ามีการสร้างเซลล์สืบพันธุ์ที่แตกต่างกันในระหว่าง 3 ระยะ และพบว่าปูนามีอสุจิในท่อสืบพันธุ์ตลอดทั้งปีแสดงให้เห็นถึงความสามารถในการสร้างอสุจิได้ต่อเนื่อง นอกจากนี้ยังพบอสุจิในถุงเก็บอสุจิ (spermatheca) ของปูเพศเมียด้วย ส่วนปูนาเพศเมียมีค่า GSI สูง 2 ช่วง คือ ฤดูแล้ง (กุมภาพันธ์) และฤดูฝน (มิถุนายน และสิงหาคม) โดยมีการเปลี่ยนแปลงสอดคล้องกับระดับฟอสโฟไลโพโปรตีนใน hemolymph รังไข่มีการเปลี่ยนแปลงสีและขนาดจากการสะสมไข่แดงและการเจริญของเซลล์ไข่ เมื่อตรวจสอบภายใต้กล้องจุลทรรศน์พบว่าสามารถจำแนกการเจริญได้เป็น 5 ระยะได้แก่ 1) resting stage 2) vitellogenic stage 3) pre-mature stage 4) mature stage และ 5) spawning and regenerating stage

ในการศึกษาเอ็มบริโอ เก็บปูนาตั้งท้องที่พบในช่วงเดือนมีนาคมและเมษายน พ.ศ.2559 แล้วสุ่มตัวอย่างไข่ปูมารักษาสภาพในเอทานอล บันทึกภาพระยะเอ็มบริโอโดยใช้กล้อง stereomicroscope ก่อนนำไข่ปูไปย้อมสี fluorescent และบันทึกภาพด้วยกล้อง confocal microscope พบว่าการเจริญของเอ็มบริโอปูนาประกอบด้วย ระยะ egg-cleavage, egg-blastula, egg-gastrula, egg-nauplius, egg-zoea และ egg-megalopa ซึ่งสามารถเจริญจนเป็นตัวสมบูรณ์ภายใน 12 วัน โดยฟักในระยะ megalopa และใช้เวลา 1-3 ชั่วโมง เพื่อ metamorphosis เป็นลูกปู ผลการศึกษาทำให้สามารถระบุระยะการเจริญปกติของปูนาน่านได้

สาขาวิชา สัตววิทยา
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ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Rachata Maneein : REPRODUCTIVE AND DEVELOPMENTAL BIOLOGY OF RICE FIELD CRAB *Esanthelphusa nani* (Naiyanetr, 1984). Advisor: Asst. Prof. NOPPADON KITANA, Ph.D. Co-advisor: JIRARACH KITANA, Ph.D.,Assoc. Prof. Julien Claude, Ph.D.

The rice field crabs are freshwater crabs that can be considered as pest in paddy field as well as a stable food source by local people. Either of these roles requires a good knowledge of its reproductive biology to manage population in nature. In this study, reproductive and endocrine cycles of male and female, and development of embryo were examined. Adult *Esanthelphusa nani* were field caught from Nan province during November 2015 to November 2016. Crabs were euthanized and dissected for reproductive tracts. Accessory organ nearby the mandibular muscle was collected for endocrine activities in male, while hemolymph was collected for vitellogenin measurement in female. Gonadosomatic index (GSI) was calculated based on the relative weight of the reproductive tract and body weight.

Male GSI showed a significant temporal difference with the peak in wet season (September). Testis can be divided into 3 stages with increasing in size from 1) resting, 2) developing, and 3) active stages. Microscopic examination revealed the different activity among three stages. Meanwhile, mature sperms can be found in male reproductive tract throughout the year, indicating prolonged spermatogenic activities. Moreover, mature sperms can be deposited and found in female spermatheca. Female GSI showed two significant peaks in dry season (February) and wet season (June and August). Female GSI profile is in agreement with levels of phospholipoprotein in hemolymph. Ovaries changed in size and color due to presence of yolk and mature oocytes. Microscopic examination of ovarian tissue showed that proportions of oocyte class in ovarian tissue could be used as an important tool for identifying ovarian cycle into five stages including 1) resting, 2) vitellogenic, 3) pre-mature, 4) mature, and 5) spawning and regenerating stages.

Ovigerous crabs found during March and April, 2016 were collected for embryonic study. Eggs were randomly sampled and fixed in ethanol. Eggs were initially examined and recorded for embryonic stage under a stereomicroscope. Afterward, fluorescent technique was applied to observe morphology and identify stage of development under a confocal microscope. It was found that the embryonic development of *E. nani* encompass egg-cleavage, egg-blastula, egg-gastrula, egg-nauplius, egg-zoea and egg-megalopa, and can be completed within 12 days. Unlike other freshwater crab, *E. nani* larvae hatched at megalopa stage and took a few hours to metamorphose to juveniles. The normal developmental stage is finally established for *E. nani*

Field of Study:	Zoology	Student's Signature .....
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		Co-advisor's Signature .....

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CHAPTER I

General Introduction

To date, more than 7,000 species of true crabs (Brachyura) have been described and classified into 98 families (Tsang et al., 2014). Depending on species, true crabs display a large variability in habitats during their life cycle and can be classified into three groups: marine, primary freshwater and secondary freshwater crabs. Marine crabs live in the marine environment from the larval stages to the latest stage of their life. Primary freshwater crabs develop in freshwater and live in the continent. Secondary freshwater crabs are transitional between these two categories with a larval stage in the sea and an adult life on land. With such environmental differences, adaptive signatures are numerous, for instance ensuring homeostasis concerning water and mineral regulation at the gill (Augusto et al., 2007). In addition, crabs have also evolved various reproductive and developmental strategies to provide the best condition for offspring to survive depending on the environment. This makes this group very interesting to study the process of adaptation in an evolution-development (evo-devo) perspective because there is a shift in environment among ontogenetic stages across groups.

In marine crab, the larva hatches as a zoeal stage and this stage is followed by four zoeae stages and one megalopa (Arshad et al., 2006). Secondary freshwater crabs similarly hatch as a zoeal stage, but their development tends to be faster comprising only two zoeae and one megalopa, indicating an abbreviation of larval development (Bolaños et al., 2005). On the contrary, the primary freshwater crab hatch directly into a juvenile, while development of the zoeae and megalopa stages occurred inside the

egg (Wu et al., 2010). These suggested that differences in ecology are accompanied by differences in reproductive strategies. Female marine and secondary freshwater crabs invest small amounts of energy into eggs, then let their free-living broods drift as plankton; they however produce a lot of eggs and display thus an *r*-strategy. In contrast, female primary freshwater crab stores large amounts of energy into eggs so that the offspring could complete larval development inside the egg. Moreover, the mother has to spend extra energy to retain hatchlings for several days before releasing them. They produce less eggs and display a *k*-strategy (Vogt, 2013). This difference is an important condition for insuring survival in different environments.

Although the structure and function of reproductive and endocrine organs are similar among crabs, variation in seasonal patterns are reported among crab species (Serrano-Pinto et al., 2005; Devi and Smija, 2014). Little is known, however, on differences among ecological groups of crabs. However, it could be very promising to compare developmental physiology among freshwater and marine crabs because the differences in the functioning of the endocrine systems among these groups could maybe explain why larval development is so different among groups (Girish et al., 2014).

The reproductive system of crab is rather well established in several key species and there are general features shared by the whole group. Male reproductive system of crabs commonly consists of a pair of testes, a pair of vas deferens (VD) and a pair of ejaculatory ducts (or posterior VD) that exists in an “H” or “X” shape (Ryan, 1967; Stewart et al., 2010; da Silva et al., 2012; Devi and Smija, 2014). Testes of crabs are tubular or lobular and, in the latter case, composed of numerous seminiferous ducts (Gupta and Chatterjee, 1976; Stewart et al., 2010). The testes are

followed by the vas deferens which locates longitudinally at the posterior region of the crab body. The ejaculatory duct is a smooth and narrow tube that extends between muscles of the fifth pair of pereiopods (legs) and connects to gonophores via a tube-like papilla penis located at the coxa base.

Female reproductive system in Brachyuran consists of an “H” or “X” shaped ovary, a pair of oviducts, a pair of spermatheca, and a pair of genital opening (Minagawa et al., 1993; Becker and Türkay, 2010; Smija and Devi, 2015). The ovary locates in cephalothorax above the hepatopancreas and can be divided into anterior and posterior horns. Left and right subunits of anterior horns are connected by central bridge or commissure in the region of the posterior cardiac stomach. A pair of oviducts links between the posterior horns and spermatheca on each side of body cavity and lateral pericardium. A pair of genital openings are formed between spermatheca and integument of the 6^{th} sternum plate.

In egg-laying animals, vitellogenin, a yolk-protein precursor, is produced in specific tissues and subsequently accumulated in the ovary before to be modified into vitellin (Meusy and Payen, 1988b). These processes are regulated via the endocrine system and the presence of yolk proteins has been frequently used to study the hormones involved in the control of reproduction (Fingerman, 1987). Vitellogenin synthesis was found to fluctuate upon stage of reproduction (Jasmani et al., 2004) and the relationship between hemolymph vitellogenin levels and ovarian development has been found (Okumura et al., 2004). Enzyme-linked immunosorbent assay (ELISA) was employed to measure levels of vitellogenin in crab hemolymph and the fluctuating vitellogenin levels in different seasons were reported (Thongda et al., 2015).

ELISA is a direct immunochemical assay targeting the interested chemical molecules by using specificity between antibody and antigen. With the difficulties in developing specific ELISA for vitellogenin, alkali-labile phosphoprotein phosphorus (ALP) has been used as an alternative method to detect levels of vitellogenin by determining the amount of phosphate linked with protein. The technique is inexpensive and not limited by species-specific antibodies and thus become a method of choice to evaluate reproductive status in female animals. There are reports that ALP strongly relates to vitellogenin in hepatopancreas, gonad and hemolymph (Aarab et al., 2004; Gagné et al., 2005; Gagné and André, 2011).

There are several major endocrine glands that play important roles in regulating reproductive development in male and female crabs including X-organ sinus glands and mandibular organs.

X-organ sinus glands (XO-SG) were firstly described by Hanström (1933). XO-SG is neuroendocrine tissue with milky whitish in appearance located between bases of eyestalks and frontal brains of the crabs (Keller et al., 1994). They can be divided into three district parts: medulla externa, medulla internal and medulla terminalis. Each part of XO-SG composes of a different cluster of beta and gamma neurosecretory cells (Enami, 1951). Removal of the XO-SG can accelerate female reproductive development including initiate ovarian maturation and vitellogenin production (Adiyodi and Subramoniam, 1983). Later studies suggested that XO-SG secretes a gonad-inhibiting hormone (GIH). GIH not only inhibits gonadal maturation but also inhibits process of vitellogenesis (Subramoniam, 2011). In male crab, the elimination of XO caused increasing testicular index (Nagaraju and Borst,

2008) and gradual reproductive growth by stimulating androgenic gland (Khalaila et al., 2002).

Other glands include mandibular organs of crabs which were firstly described by Le Roux (1968). This pair of endocrine glands with pale yellowish in appearance connects to anterior part of the mandibular tendons and posterior part of the mandible. Previous studies of MO revealed that the gland composes of glandular epithelium surrounded by connective tissue layer (Yudin et al., 1980). MO plays an important role in regulating reproduction (Reddy and Ramamurthi, 1998), and molting (Tamone and Chang, 1993). In *Carcinus maenas*, MO was gradually active during ovarian development (Hinsch, 1980). In *Oziotelphusa senex*, its MO increased the size up to two times during ovarian maturation (Nagaraju et al., 2004). Immature female crabs injected with MO extract could initiate ovarian development (Hinsch, 1980). Methyl farnesoate (MF) has been identified as secretory chemical of the MO. The chemical structure of MF is nearly identical to that of juvenile hormone III (JH III) in insects (Laufer et al., 1987) as injection of JH III into female crabs also resulted in ovarian growth (Hinsch, 1981). Several studies have suggested that MO is controlled by neuropeptide from X-organ-sinus gland as shown in rising level of MF in hemolymph after eyestalk ablation (Borst et al., 2002). *In vitro* studies showed that MF stimulated oocyte size increase and hepatopancreas vitellogenesis (Borst et al., 2002).In male crab, the secretion of MO also involves on gonadal development (Chen et al., 2004).

In Thailand, freshwater crabs are diverse and can be classified into three families including Parathelphusidae (27 species), Potamidae (38 species), and Gecarcinucidae (7 species) (Ng and Naiyanetr, 1993). Still few studies have been done on the ecology and developmental biology of these species, and more generally,

the development of primary freshwater crabs has been reported just for a few species. However, better documenting the development and reproductive strategy of these crabs can help to better understand the adaptive radiation of the whole group and the transition from marine to freshwater environment. In this study, a primary freshwater crab *Esanthelphusa nani* (Naiyanetr, 1984) in Family Parathelphusidae was used as a representative species. In Thailand, this family contains 27 species including 17 species of rice field crabs: the genus *Sayamia* (4 species), the genus *Chulathelphusa* (2 species) and the genus *Esanthelphusa* (11 species).

The rice field crab *Esanthelphusa nani* was firstly described based on specimens caught at Nan Province (Naiyanetr, 1984). In some aspects, rice field crabs are considered as pests that destroy rice production of rice field in Thailand. This problem always occurs at the beginning of rice farming in rainy season that the crab is starting to store energy for hibernation in dry season. At the same time, rice field crabs have been used as food resources by local people in Thailand and some countries in Asia (Rajasekaran and Whiteford, 1993). Regulating the reproduction of this crab also requires a good basic knowledge of its reproductive biology to control (increase/ decrease) population in the natural habitat.

To gain insight into reproductive and developmental biology of this freshwater crab, the research scheme shown in Figure 1.1 was carried out. The objectives of the study are as follows:

1. To document the reproductive strategy of *E. nani* by:

1.1. Determining gonadal cycle of the adult male and female rice field crab, *E. nani*, based on seasonal changes in morphology and histology of the reproductive system.

1.2. Determining reproductive endocrine cycle of the adult male and female *E. nani* based on seasonal changes in morphology/histology of endocrine organs and corresponding secretory activities.

2. To describe, examine and compare the somatic embryonic development of *E. nani* based on changes in morphology.

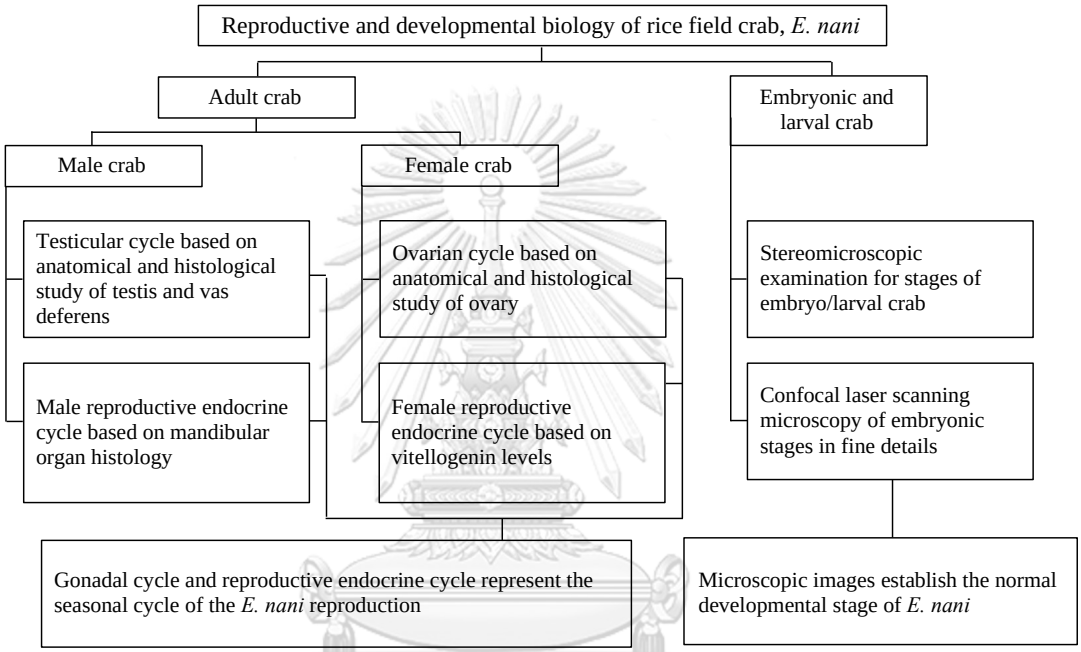


Figure 1.1 Research framework for reproductive and developmental biology of rice field crab *E. nani*

CHAPTER II

Literature Reviews

This part attempts to gather the information regarding 1) evolutionary relationships within Brachyura, 2) their general morphology, and 3) their reproductive biology. In particular, the review focused on male and female reproductive systems, as well as on the hormonal control in these reproductive systems. Finally, the review will gather information regarding the embryonic development of crabs and relatives as well as the definition of larval stages in Brachyura.

Phylogenetic relationship of brachyuran: the presence of freshwater crab

Brachyura infra-order correspond to true crabs that are the most diverse groups of crustaceans with 98 families and more than 7,000 species described. They live in diverse environment including marine, estuary, freshwater, and terrestrial habitats (Ng et al., 2008; De Grave et al., 2009). The evolutionary relationships among the brachyuran families have been poorly understood until the use of molecular methods (Ahyong et al., 2007). Brachyura has been divided into three sections including Podotremata, Heterotremata, and Thoracotremata based on the morphology of the gonopore (Guinot, 1979; see section regarding the morphology of crabs and sex organs). Podotremata is often said primitive because they maintain potentially ancestral characters, whereas the Heterotremata and Thoracotremata sections form the Eubrachyura (true crabs). The monophyly of Eubrachyura is recognized based on gonopore and spermatheca (Guinot et al., 2013), however, Podotremata's monophyly is controversial. Indeed, the group is, mostly, presenting plesiomorphic and controversial synapomorphic characters (Scholtz and McLay

2009). Podotremata's monophyly, in addition, has not been verified by the use of molecular phylogenetic analyses (Ahyong et al., 2007). Ahyong et al. (2007) proposed splitting the former Podotremata into three clades: Dromiacea, Raninoida, and Cyclodorippoida. Further morphological research found agreement with these divisions (De Grave et al., 2009; Scholtz and McLay, 2009). Moreover, the relationship between Heterotremata and Thoracotremata in Eubrachyura has been not clear for a long time, especially whether both parts are monophyletic or whether Thoracotremata derives from Heterotremata (Karasawa et al., 2011).

Of particular interest in this context are the origins of the families of primary freshwater crabs within Heterotremata. These families are Pseudothelphusidae, Potamonautidae, Potamidae, Gecarcinucidae, and Trichodactylidae. Primary freshwater crabs live exclusively in freshwater or on land and never enter brackish or coastal waters for breeding (Cumberlidge et al., 2009). This ecological group submits direct growth and complete their aquatic life cycles. More than 1,300 species of primary freshwater crabs are recognized with frequent discovery of additional species. This highly diverse group and particular ecology have received interest recently (Cumberlidge et al., 2009; Klaus et al., 2009). Although alpha systematics is relatively well assessed, the higher relationships among freshwater crabs are still controversial. The five families typically could correspond to two or more distinct evolution to freshwater: the Trichodactylidae of South Americas and a potentially monophyletic group composed of the other four families distributed around the world. Many morphological characteristics would suggest a strong relationship between Trichodactylidae and Portunoidea (von Sternberg and Cumberlidge, 2001, 2003). Molecular analyzes do not, however, recover this relationship (Schubart and

Reuschel, 2009). These groups are generally considered as heterotremes (Ahyong et al., 2007) but several authors claim that they share a certain amount of thoracotrem synapomorphic (von Sternberg and Cumberlidge, 2001). A morphological cladistic analysis also suggested that Thoracotremata could include the non-trichodactylid freshwater crab group of sister marines and the two groups may come from xanthoid-like (von Sternberg and Cumberlidge, 2001). Considering the circum tropical distribution of non-trichodactylid freshwater crabs, a single evolutionary source suggests that the group's diversification and radiation preceded the Gondwana or Pangaea (~200 Ma) breakup (Rodriguez and Ng, 1995). In this case, tectonic activities would have been tracked by subsequent cladogenesis arising from the Gondwana split. Nevertheless, this phylogenetic theory implies an ancient source for freshwater crabs and the remaining Eubrachyura, which is not confirmed by the fossil record (Klaus et al., 2009). In summary, the origin of the primary freshwater crabs, the status and relationship of heterotrems in eubrachyuran sections have long time been the objects of debates. This situation changed recently with the comprehensive phylogenetic analyses of Tsang based on molecular data (Tsang et al., 2014) (Fig. 2.1). When mapped on phylogeny, it is obvious that primary freshwater lifestyle evolved independently three times in Heterotremata and that secondary freshwater lifestyle evolved independently in Heterotremata and Thoracotremata. Better documenting freshwater crab morphology, development and reproduction across these groups can therefore be particularly interesting to understand common patterns of convergence in that new phylogenetic context.

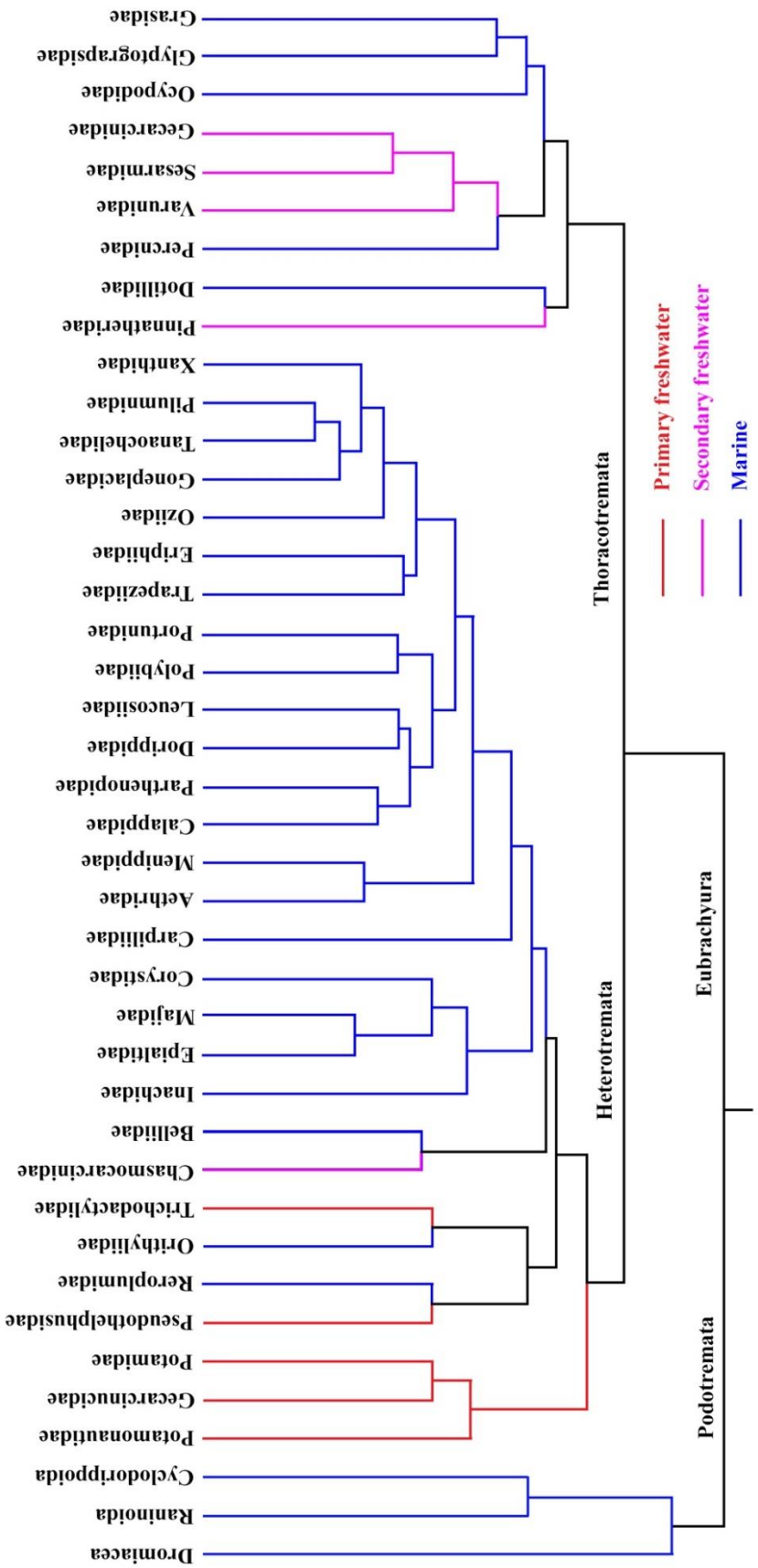


Figure 2.1 Phylogenetic relationships between members of the brachyura family; modified tree based from analyses of genetic data sets by Tsang et al. (2014). The different color presents the three major ecological lifestyles: primary freshwater, secondary freshwater and marine crabs

General morphology of crabs

Like other arthropods, brachyurans have a body composed of series of metameras organized in different tagmas ranging from anterior to posterior. Once fusion takes place, joints between some metameras can be lost, as in the case of cephalic somites in crustaceans (Gruner and Scholtz, 2004). During development, limb differentiates according to their position and derived from the typical biramal arthropod appendages. Appendages can be grouped according to their function (their respiration, feeding, locomotion, etc). The brachyuran body plans, according to appendage and segment is shown in Table 2.1.

Table 2.1 Layout of the body segments (derived from somites) and appendages of a brachyuran (Based on Scholtz (2001))

Brain and eyestalk				
CEPHALOTHORAX	TAGMA CEPHALON		antennule	Somite 1
			antenna	Somite 2
			mandible	Somite 3
			maxillule	Somite 4
			maxilla	Somite 5
	TAGMA THORAX		1st maxillipeds	Somite 6
			2nd maxillipeds	Somite 7
			3rd maxillipeds	Somite 8
			1st pereiopods	Somite 9
			2nd pereiopods	Somite 10
			3rd pereiopods	Somite 11
			4th pereiopods	Somite 12
			5th pereiopods	Somite 13
ABDOMEN	TAGMA PLEON		1st pleopods	Somite 14
			2nd pleopods	Somite 15
			3rd pleopods	Somite 16
			4th pleopods	Somite 17
			5th pleopods	Somite 18
			6th pleopods	Somite 19
Telson				

The anterior extension of the head is the acron which consists of brain and eyestalk. The anterior appendages are the cephalic somites, consisting of antennule, antenna, mandible, maxillule, and maxilla. The next tagma consists of thorax somites which develop to be thoracopod appendages including three pairs of maxillipeds, five pair of pereiopods. Mandible, maxillule, maxilla, and the three pairs of maxillipeds ensure the feeding function. The abdominal somites consist of six pair of pleopods and six abdominal plates. The telson is a terminal somite without appendages.

The carapace is a dorsal shield that is often extended laterally. Most authors use the term "cephalothorax" to refer to the whole structure of the fused cephalic and thoracic upper somites in a single dorsal shield. The carapace shape is usually different from species to species, allowing to produce diagnostic characters. The carapace surface can be marked by grooves associated regions. Different systems for naming carapace regions have been established which often differ significantly among various crab groups. In this thesis, the following nomenclature based on internal organ is used for the rice field crab, *E. nani*: including orbital, epigastric, left and right protogastric, mesogastric hepatic, branchial, gonad, cardiac, and intestinal regions (Guinot, 1979) (Fig. 2.2).

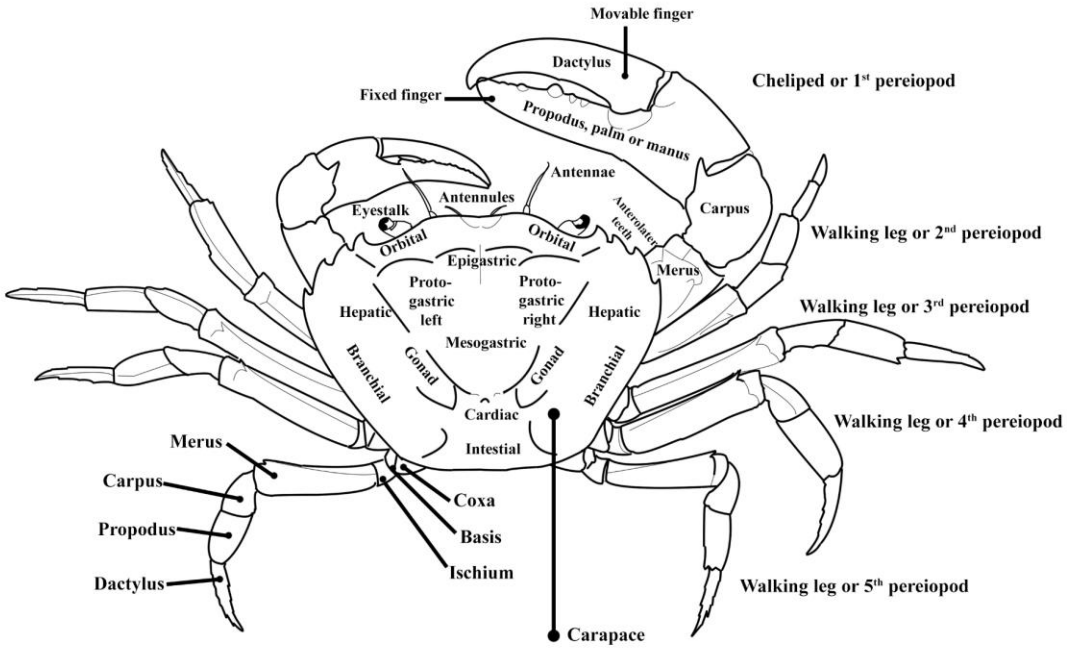


Figure 2.2 General terminology for cephalothorax and internal organ regions of brachyurans (shown on *E. nani*)

The five pairs of pereiopods (the ten legs defining decapopda within crustacea) are organized as a pair of cheliped, corresponding to the 1st pereiopods and four pairs of walking legs corresponding to pereiopods 2 to 5. Pereiopods commonly consist of six segments which are articulated by a pair of external condylar joints to allow the legs moving in various directions. Like other arthropods, the six segments are named: coxa at leg base, basis, lschium, merus, carpus, propodus, and dactylus (Vidal-Gadea and Belanger, 2013). The first pereiopod is called a cheliped because it presents claw or chela. The chelipeds do not only develop and associate with feeding, but also associate to defense, intraspecific aggression or courtship behaviors in many species. The chelipeds consist of coxa, fused basis and ischium part, elongated merus, short and rounded carpus, propodus that transforms into a palm (manus) or fixed finger, and dactylus that transforms into a pincer or movable finger. The palm and pincer are usually referred to the chela. Generally, podotremes have similar left and

right claw size. Heterochelous character is a character resulting from antisymmetry developed in males (not in females). It is characterized by the presence of a big claw on one side which is a sexual dimorphic trait in most crabs, especially in genus *Uca*. The larger claw may be associated with sexual selection such as catching female for maturing, showoff in courtship behaviors, pre- and post-copulatory or brood care. While the small claw usually plays important role in the feeding mechanism.

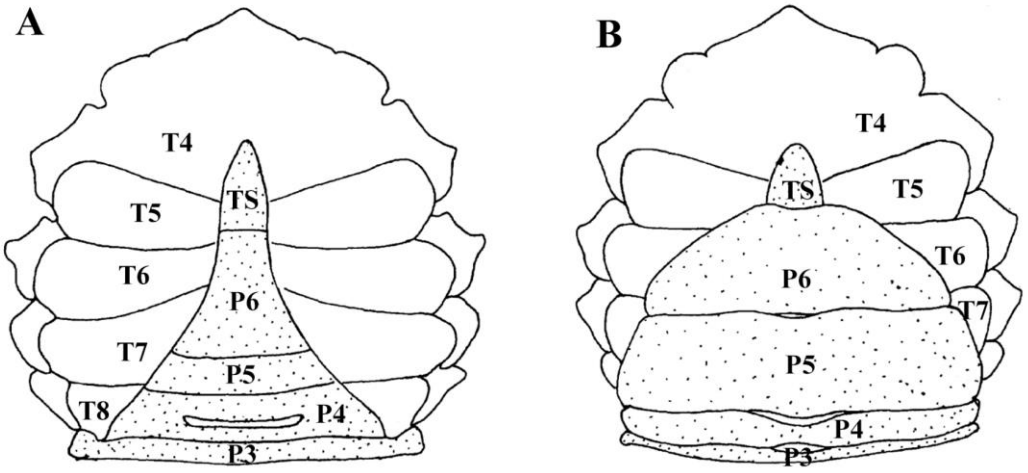


Figure 2.3 Eubrachyuran: ventral view of thoracic sternal plate and abdominal plates. A=male; B=female; P3-P6: 3rd -6th abdominal plates; T4-T8: 4th -8th sternites; TS: telson (modification based on Anger (2001)).

On the ventral side, eight thoracic somites form the sternal plate found in Decapoda. There is a number of important taxonomic characteristics that relate the shape and the degree of fusion between the somites. The sternal plate could vary considerably in width between very narrow and extremely broad in some crabs (Guinot, 2011). Sternites 1st -4th are fused in various degrees and form an anterior "triangle" that extends between the third maxillipeds and the oral cavity. Because of sternite 4 is the longest of those, only this one is well visible externally (Fig. 2.3). The 4th to 8th sternites present externally a part of the sternal plate called “episternites”.

The partition between episternites usually present the position of the pereiopod. In some crabs, the 8th sternite could be rudimentary or absent. The sterno-pleonal cavity is covered forwardly by the abdominal plate or pleonal plate from the 8th sternite to at least the posterior edge of 4th sternite.

In general, Brachyurans have six pleonal somites and one telson which are fused to be an abdominal plate. The P3-P6 abdominal plates are often visible externally, whereas the P1-P2 are hidden by the carapace (Fig. 2.3). The shapes of abdominal plate between male and female crabs exhibit important sex dimorphism. The P3-P6 abdominal plate of male crab commonly forms a narrow abdominal shape (Fig. 2.3A). In the sterno-pleonal cavity, male crabs have the first two pairs of uniramous pleopods modified as two pair of gonopods, specifically used in copulation and sperm delivery (Fig. 2.4D). The first gonopod (G1) was longitudinally folded to form a cylindrical tube encasing an ejaculatory duct or penis. The second gonopod (G2) form whip-like shape which is smaller than the G1. The G2 fits into the inner channel of the G1 folds and serves a purpose of sperm transportation or pumping from the G1 into the gonopore opening of female crabs.

Female pleons are broadly expanded for the carriage of eggs that locate over the sternum (Fig. 2.3B). Females can carry large egg masses and possess well-developed and modified abdominal plate that is called a brood pouch. In the brood chamber or sterno-pleonal cavity, the abdominal plate is able to be lifted for creating a branchio-sternal channel between pleopods that allows the oxygenation of the eggs (Drach, 1955). The unmodified pleopods of Eubrachyura female are present at pleonal somites 2-5 (the first still present in podotremes). The pleopods mainly function consist of the egg holding and care.

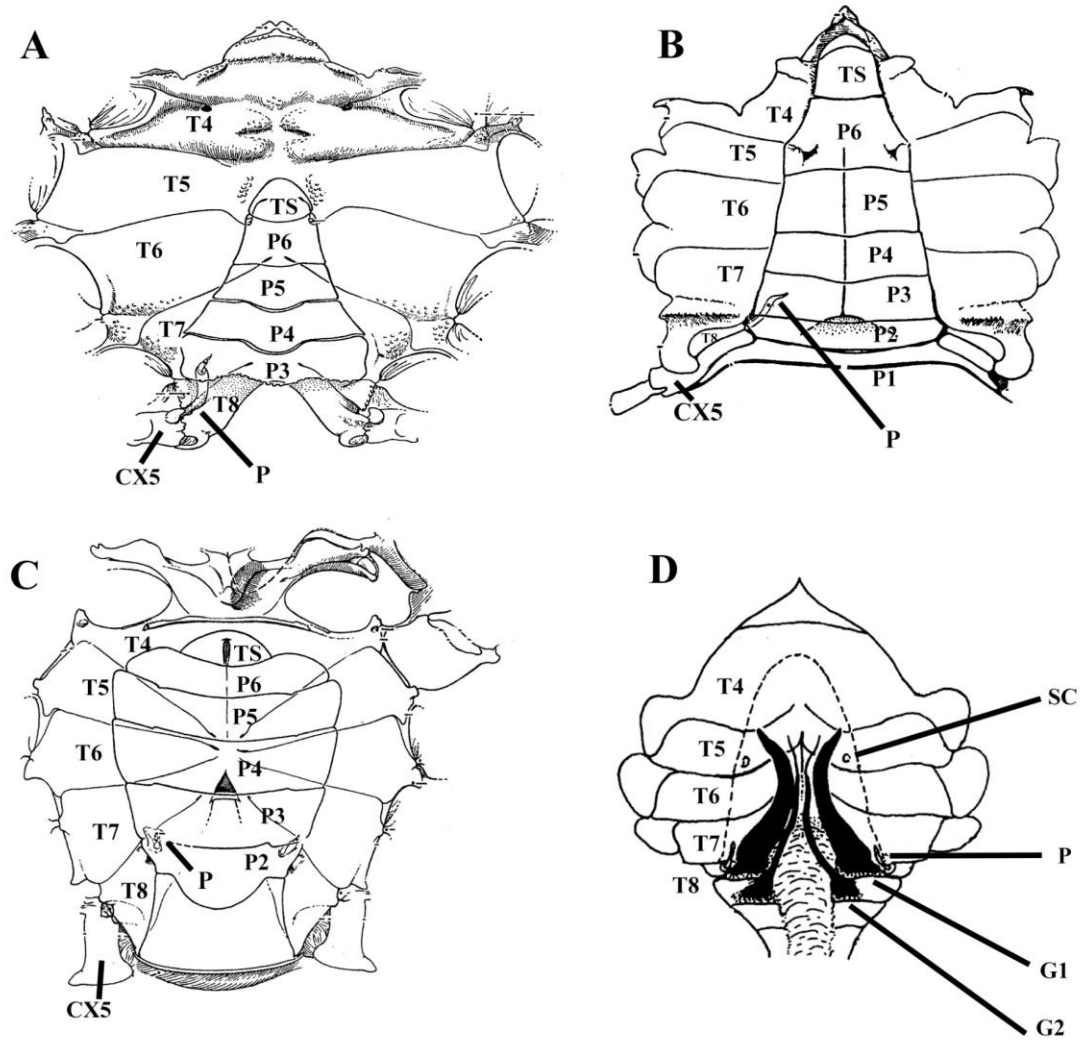


Figure 2.4 Male thoracic sternums and pleons in Brachyura. A) *Medorippe lanata* (Podotremata); B) *Palicus caronii* (Heterotremata); C) *Mictyris longicarpus* (Thoracotremata); D) Ventral view of cephalothorax show sexual organ in site sterno-pleonal cavity. G1: first gonopod; G2: second gonopod; P1-P6: 1st -6th abdominal plates; T4-T8: 4th - 8th sternites; TS: telson; SC: sterno-pleonal cavity (modification based on Guinot (1979)).

Male penis and female gonopore openings are the crucial sexual characteristics to classify Brachyura into Podotremata, Heterotremata, and Thoracotremata (Guinot, 1978). In Podotremata, male penis and female gonopore locate on coxa in both sexes (Fig. 2.4A presents male, Fig. 2.5B presents female). The male penis forms at the fifth coxa of pereiopod, while the female gonopores open at the third coxa of pereiopod. The spermatheca opening is separated from gonopores

and located on the 6th sternite plate (T6). In heterotremata, male penis is localized on the fifth coxa of pereiopod, whereas the female gonopore openings located on the 6th sternite plate (T6) (Fig. 2.4B presents male, Fig. 2.5C presents female). The fusion between gonopore and spermatheca opening may present a significant synapomorphy for Eubrachyura as this character is also found in Thoracotremata (Fig. 2.5C). Thoracotremata male penis however differs in terms of location by comparison to Heterotremata. Indeed, in Thoracotremata, this organ develops onto the 8th sternite (Fig. 2.4C). Both penis and gonopore opening on the sternite is a synapomorphic character of Thoracotremata.

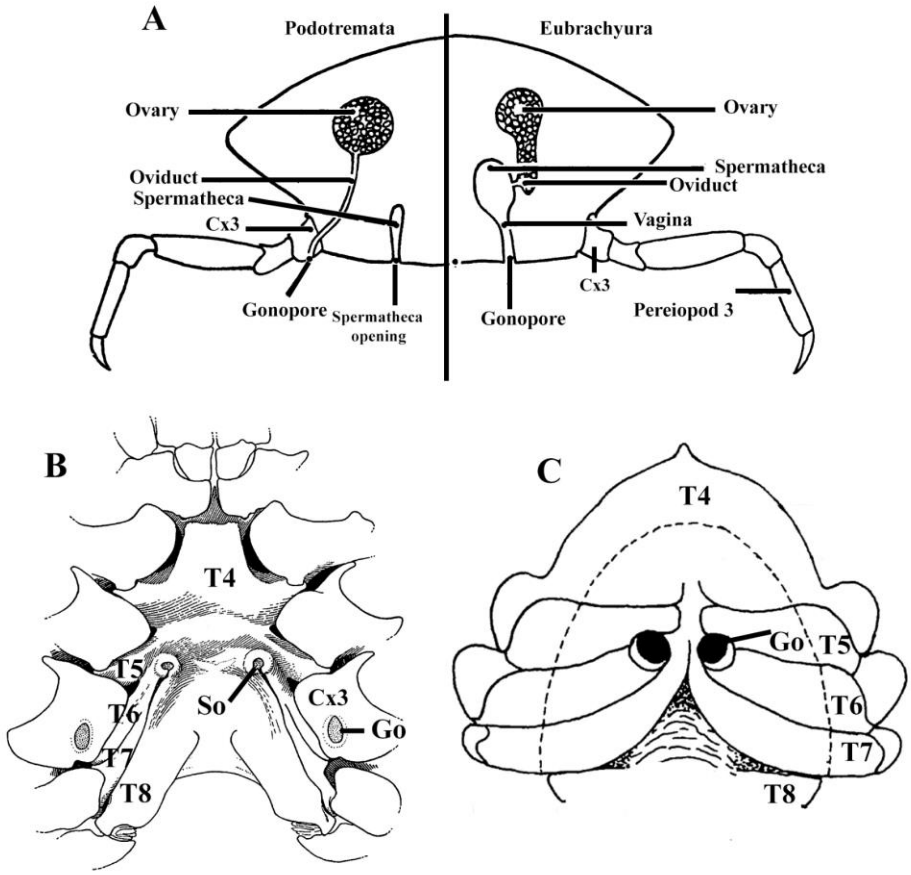


Figure 2.5 A) Comparative diagram of reproductive duct pattern between female Podotremata (left) and Eubrachyura; B) Famale thoracic sternums and sterno-pleonal cavity of *Stimdromia lateralis* (Podotremata); and C) Eubrachyura. (modification based on (Guinot, 1979))

Male internal reproductive tract

Anatomy of male reproductive tract has been documented for many years. *Homolodromia kai* (Homolodromiidae, marine crabs) and *Sphaerodromia lamellata* (Dromiidae, marine crabs) (Guinot et al., 1998) have been documented as representative of Podotremata. *Callinectes sapidus* (Epialtidae, marine crabs) (Johnson, 1980) *Portunus sanguinolentus* (Portunidae, marine crabs) (Ryan, 1967) and *Chionoecetes opilio* (Oregoniidae, marine crabs) (Sainte-Marie and Sainte-Marie, 1999) have been documented for Heterotremata. *Goniopsis cruentata* (Grapsidae, mangrove crabs) (Garcia and Silva, 2006) and *Ucides cordatus* (Ocypodidae, mangrove crabs) (Castilho et al., 2008) have been documented for Thoracotremata.

Based on this work, the general morphology can be described as follow: a pair of testes normally locates anterior-dorsally in the cephalothorax, above the hepatopancreas. The testis consists of coiled and convoluted seminiferous tubules. These seminiferous tubules can show three district zones: the germinal zones that are important for producing spermatogonia, the transitional zones zone correspond to the differentiation and maturation of gametes and evacuated zones connect to vas deferens to ensure the transport of spermatozoa and seminal fluids. The end of testis merges into a complex, coiled, and looped vas deferens, usually divided into three different regions. The anterior vas deferens (AVD) locates next to the testis near the mid-line, back to the foregut and is usually a long, thin tubule that is highly convoluted. The main role is to collect spermatozoa from evacuated zones of testis and to enclose the spermatozoa into spermatophores. Middle vas deferens (MVD) locates at the ventral testis, alongside the gut, and is typically the most visible part of the mature male reproductive tract. It is a storage site of spermatophores and globules

of seminal fluid until needed for copulation. Posterior vas deferens (PVD) is associated with ejaculation. Its function is to send the reproductive seminal fluid and sperm into the female crab. A pair of gonopore of podotremes and heterotremes opens at the ventral coxae of the fifth walking leg, while in thoracotremes the gonopores open at of the 8th thoracic sternite. External extension of the ejaculatory gonopore forms a pair of penes or the first gonopod. It is generally short, but some species can be very long (Ng et al., 2008). Normally, the gonopore is protected in a sternal groove. Spermatophores are transported into the female spermathecae by synchronized actions of G1 and G2.

Female internal reproductive tract

The female reproductive tract morphology is documented in *Ranina ranina* (Raninidae, marine crabs) for Podotremata (Minagawa et al., 2013). *Uca rapax* (Ocypodidae, mangrove crabs), *Portunus sanguinolentus* (Portunidae, marine crabs) and *Sinopotamon henanense* (Potamidae, freshwater crabs) for Eubrachyura (Castiglioni et al., 2007; Soundarapandian et al., 2014; Sun et al., 2018).

A pair of ovaries always found on the hepatopancreas in the cephalothorax. Depending on crab species, the ovaries of mature female crabs are small or large enough to obscure the other organs in the cephalothorax, especially when they fully developed. The ovary is usually divided into an anterior and posterior region. A thin commissure linking the right and left ovaries in the midline result in an H or X-shaped appearance. During ovulation, the mature eggs are carried out by the oviduct. The Podotremata usually has elongated oviducts which open at gonopore openings on the coxa of 3rd pereiopod (Fig 2.5A). Once the mature eggs are transferred into the sterno-pleonal cavity, the female release the stored sperms in spermatheca at 6th sternite into

the sterno-pleonal cavity to parachive fertilization. In contrast, the Eubrachyura has short oviducts and openings into spermatheca atrium where is the fertilization take place. The fertilized eggs are carried pass though the spermatheca tube that commonly called “vagina” and exit at the gonopore openings. The oviduct and spermatheca have two distinct developmental origins and this is documented in Guinot et al. (2013).

Hormonal controls on sexual reproduction in crabs

The hormone regulation of reproduction is well documented in Decapoda. A variety of neuroendocrine hormones play a crucial role in regulating gonad maturation (Nagaraju, 2007; Mazurová et al., 2008; Raviv et al., 2008) (Fig 2.6). Two antagonistic neuropeptides, the gonad inhibiting hormone (GIH) and the gonad stimulating factor (GSF) act as regulators of gonadal maturation in crustaceans. In female, GIH is also called vitellogenesis inhibiting hormone (VIH). GIH is a neuropeptide hormone from the X-organ–sinus gland complex of the eyestalk (XO–SG), whereas GSF is a hormone produced by the brain and thoracic ganglions. Crustacean hyperglycemic hormone (CHH) synthesizes and activates from the that by eyestalk–XO–SG complexes. Since the CHH receptor has been reported in many organs (Webster, 1993; de Kleijn et al., 1995; Gu et al., 2002; Zmora et al., 2009), this neuropeptide hormone does not directly influence gonad maturation, but is able to interact with other organs such as mandibular organ, androgenic gland, y-organ, brain, thoracic ganglion, hepatopancreas, etc. The mandibular organ inhibiting hormone, a hormone of XO–SG, indirectly impact reproductive maturation both in female and male via repressing methyl farnesoate synthesis in the mandibular organ (Wainwright et al., 1996).

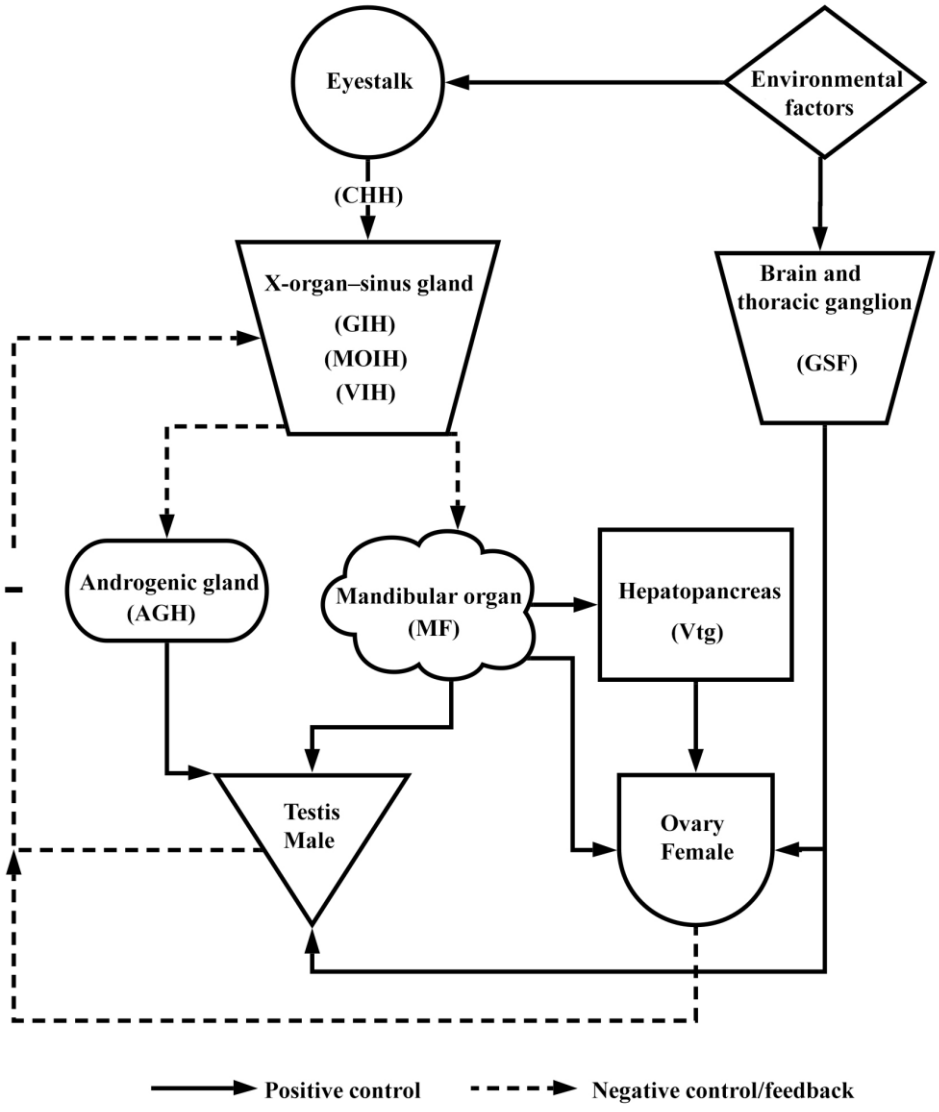


Figure 2.6 A flowchart shows summary of hormonal controls on sexual reproduction in crabs. The positive controls present in solid lines, whereas the negative controls or feedbacks present in dash lines.

Malacostracan crustaceans have a specific endocrine gland called “androgenic gland, AG” which is unique in male. GIH and GSF are respectively inhibitor and activator of AG growth and its hormonal product, the androgenic gland hormone (AGH). AGH plays a key role in regulating spermatogenesis in testis of male crustaceans, as well as for influencing the maturation of sex organs and secondary sexual characters (Fig 2.6). Charniaux-Cotton (1962) firstly described the function of

AG in terms of growth and maturation controls of the crustacean male reproductive and secondary sexual characteristics. Furthermore, when the AG of male crustaceans are ablated, spermatogenesis is terminated (Nagamine et al., 1980). On the other hand, eyestalk ablation, which removes the preliminary signal of GIH, leads to hypertrophy of AGs and a gradually increasing spermatogenesis (Liu et al., 2008). Additional GSF is capable of causing AG on spermatogenesis. AGH was isolated and structurally characterized showing it comprised glycosylated dimeric peptides (Okuno et al., 2002a). Injection AGH into female result in Vtg synthesis inhibition (Chang and Sagi, 2008). Similarly, AG transplantation into female crab *Scylla paramamosain* prevented development of ovaries (Cui et al., 2005).

The mandibular organ (MO) is a pair of ductless glands which develop from ectoderm. It locates at the anterior part of the mandibular tendon base and at the posterior part of the mandible in decapod crustaceans (Yudin et al., 1980). Mandibular organs of crabs were firstly described by Le Roux (1968). Methyl farnesoate (MF) has been identified as secretory chemical of the MO. MF chemical structure is nearly identical to the well-known juvenile hormone III (JH) family, only differing in the presence of an epoxide moiety at the terminal end (Laufer et al., 1987). Many studies have suggested that MO is controlled by the neuropeptide, MOIH produced from XO–SG. Eyestalk ablation result in rising MF level in hemolymph (Borst et al., 2002). The mode of MF action directly regulates the reproductive maturation of both male and female crab.

In male, the study of MF effect on spermatogenesis is, however, limited. The AG of male usually pick up to be the case study for effect on spermatogenesis (Chung et al., 2011). However, it has been shown that the MO of male crustacean is larger

size in adult males comparing to those in juvenile (Nagaraju et al., 2004). The correlation between MF levels in hemolymph and gonad maturation has been also documented in *Libinia emarginata* (Sagi et al., 1993). Increasing MF levels is also associated with mating behavior. Male crab with higher MF level actively courts and mates with female crabs, whereas male with lower MF levels refuses this behavior (Laufer et al., 1994). Furthermore, in the crabs *Oziothelphusa senex* and *Carcinus* maenas, MF injection resulted in an increase of testicular index (Kalavathy et al., 1999; Nagaraju and Borst, 2008).

On the other hand, MO has also been studied on female crustaceans. The secretion of MO, methyl farnesoate, is able to promote the ovarian maturation in many crustaceans. Histological studies of MOs from *C. maenas* and *L. emarginata* suggest that they are more active during ovarian maturation (Le Roux, 1968; Hinsch, 1980). MO extracts injected into immature spider crabs *L. emarginata* boosts ovarian maturation (Hinsch, 1980). Eyestalk ablation increases the level of MF and the ovarian index in *L. emarginata* (Laufer et al., 1987) since the major inhibitions has been removed (Que-Tae et al., 1999). *In vitro* studies showed that oocyte growth and vitellogenin (Vtg) level in hepatopancreas and ovary were induced by MF (Ōtsu, 1963).

Vitellogenin, an essential pressure of oocyte development

Alike other oviparous species, vitellin (Vn) is the main yolk protein in crustacean oocytes that supports the growth and development of embryos and early free larval stage in providing amino acids, lipids, carbohydrates, carotenoids, and minerals (Khalaila et al., 2004). Vitellogenin (Vtg), the vitellin precursor, is synthesized inside or outside the ovary and is delivered by the hemolymph into

recipient-mediated endocytosis in the rising oocytes (Eastman-Reks and Fingerman, 1985).Vtg initiates proteolytic process and develops into several subunits in the oocytes (Lee and Watson, 1995). The synthesis and accumulation of vitellogenin are therefore essential for the growth of oocytes and embryos (Meusy and Payen, 1988a; Tsukimura, 2001).

Numerous researches focused on the gene structure and expression of vitellogenin decapods (Charniaux-Cotton, 1985; Kung et al., 2004). cDNAs encoding Vtg have been identified in shrimps and crayfishes (Abdu et al., 2002; Okuno et al., 2002b; Kung et al., 2004). Similar reports have been done on the red crab *Charybdis feriatus.* In that case, cDNAs encoding Vtg have a length of around 7 to 8 kb and cover over 2500 amino acid residues, with a significantly conserved structure, especially at their amino terminus (Mak et al., 2005). Immediately after their synthesis in extra ovarian tissues, the crustacean Vtgs are digested enzymatically and further cleavages take place in the hemolymph or the ovary (Okuno et al., 2002a; Mak et al., 2005). Vtgs of insects are separated into subunits before they are deposited into the hemolymph (Chen et al., 1999). Only after entering the developing oocytes, Vtgs are enzymatically converted to smaller yolk, lipovitellins, and phosvitins in vertebrates (Polzonetti-Magni et al., 2004). In vertebrates, Vtgs are usually synthesized by extra ovarian tissues like the liver (Byrne et al., 1989). The intestine for nematodes and the fat body for insects have been described as the respective synthesized organ for these groups (Sappington and Raikhel, 1998). In the same respect and at higher taxonomic levels, Vtg synthesis sites have been documented in crustaceans in different species. Crustacean Vtg was expressed primarily in ovary and

hepatopancreas (Tsutsui et al., 2000; Tsang et al., 2003; Jasmani et al., 2004; Kung et al., 2004; Mak et al., 2005).

Early development of arthropod

Arthropods diversity is also reflected by the diversity of their ontogenies, and also often related to the different lifestyle. Obviously, the different structure of the adult body forms in arthropods arises from very different ontogenies (Scholtz, 2005).These ontogenetic variations across group involve variation in gene expression, cleavage and gastrulation to formation and creation of germ bands, segmentation, and morphogenesis (Scholtz, 1998; Akam, 2000; Hughes and Kaufman, 2002). Likewise, postembryonic development explores all kinds of growth patterns, both direct and indirect, and a wide range of larval forms with a wide variety of life styles comparable to the adult diversity.

In several animals (cnidarians, ctenophores, annelids, mollusks, echinoderms, and chordates), the development start first by total (or holoblastic) cleavage and this early development is regarded as the most common form of cleavage in metazoan (Siewing, 1979). The blastomeres issued from the first division form later a central cavity, consisting of a liquid blastocoel, and the overall stage is called a blastula (Fioroni, 1970; Gilbert and Epel, 2015).

In most arthropod cleavage is a meroblastic cleavage mode, which is defined as a superficial cleavage (Fig. 2.7). This cleavage mode is distinguished from total cleavage by the absence of cytokinesis, resulting in the lack of cell membranes between the cleavage products. The energids are therefore incorporated in the yolk and form a polynuclear cell. After massive divisions, the energids migrates to the surface of the egg, creating an acellular blastoderm (periblastula) that starts is

cellularization by forming a membrane embracing the central yolk mass (Gilbert and Epel, 2015). At first, the total cleavage should be considered a part of cleavage at early blastoderm formation of arthropod for instance, *Achaearanea tepidariorum* (Kanayama et al., 2010), and *Drosophila melanogaster* (Campos-Ortega and Hartenstein, 2013). Later on, the composition of the central yolk mass varies. It can be either cellular or acellular, homogeneous mass or contain partitions (Fioroni, 1970). In some of crustaceans, the blastomere membranes do not pass the middle of the yolk and only partially separate the egg (Scholtz, 2005). The incomplete penetration of yolk by cleavage membranes was also considered by the same term (Fioroni, 1970).

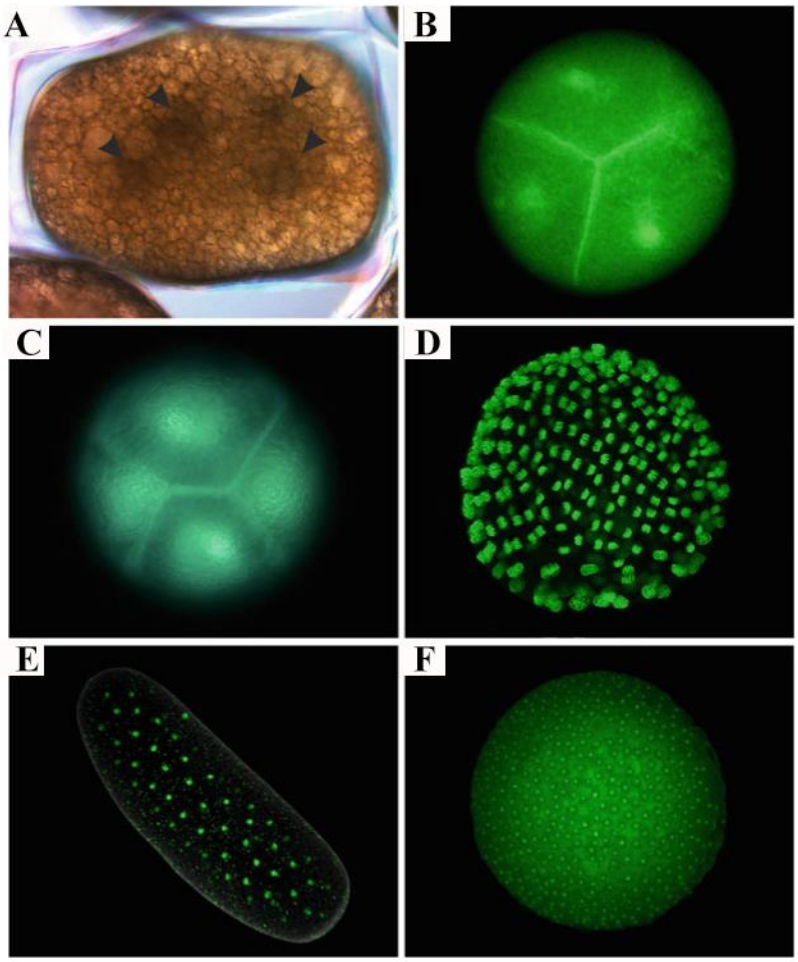


Figure 2.7 Examples of cleavage and blastoderm stage. A) superficial cleavage of isopod crustacean *Porcellio scaber*; B) Total cleavage. The four-cell-stage of the pycnogonid *Endeis spinosa*; C) Total cleavage. The four-cell-stage of the euphausiacean crustacean *Meganyctiphanes norvegica*; D) Blastoderm stage of the euphausiacean crustacean *Meganyctiphanes norvegica*; E) Superficial cleavage in *Drosophila melanogaster*; F) The blastoderm stage of the spider *Cupiennius salei* (modification based on a review of Scholtz and Wolff (2013))

Larval developmental stage in marine and estuary crab.

The most primitive, widespread, and known pattern of planktonic larval form among the crustaceans is nauplius *sensu lato* (Dahms, 2000; Scholtz, 2000). The nauplius comprises only three pairs of cephalic appendages, including the first antennae antennules, the second antennae, and the mandibles (Fig.2.8A). The larval limbs have roles primarily for feeding at nauplius. In the later stages, these appendages, lose their locomotory function and have sensory and food handling roles respectively (Anger, 2001). Nauplius stages are found in different benthic crustacean taxa such as cirriped and dendrobranchiate shrimp, but planktonic larvae are no longer present in Brachyura.

In the marine Brachyura, the ancestral and dominant type of larval development is extended by an intense series of planktonic larvae (Anger, 2001). These free larval stages follow stages of embryogenesis in the egg (Scholtz and McLay, 2009). Egg numbers per clutch are thousands to hundreds of thousands or millions and vary among crab species (Batoy et al., 1987; Smith and Hines, 1991; Leme, 2004; Albelali et al., 2010). The larval period of Decapoda is generally divided into three phases with a varying number of stages, nauplius phase, zoea, and decapodid phases followed by the juvenile period. In brachyurans, they specifically present two planktonic phases: zoea phases with a varying number of stages and one decapodid phase. The decapodid is commonly called megalopa in crabs more specifically (Anger, 2001).

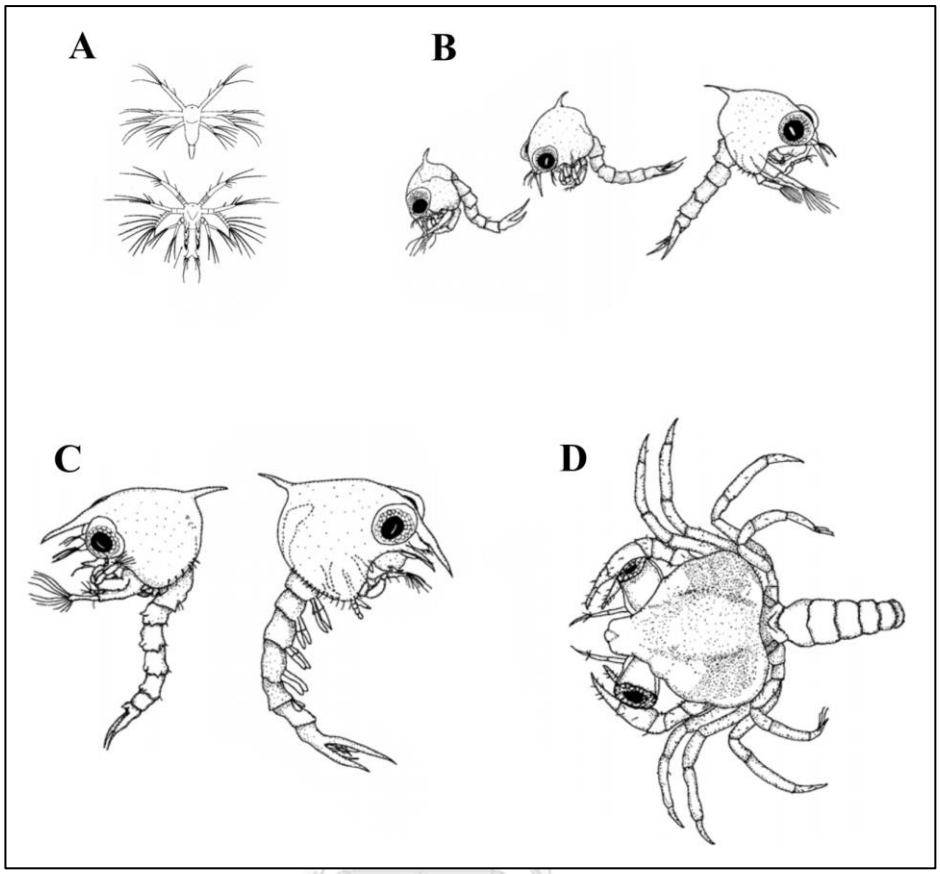


Figure 2.8 A) Free-living larvae of dendrobranchiate shrimps at nauplius stage; B-C) Free-living larvae of crab, *Uca thayeri*; B) Early zoea stages at I-III; C) Later zoea stages at IV and V; D) the megalopa stage. (Dobkin, 1961; Anger et al., 1995)

The zoea phase is the next phase following nauplius. The "primitive" Decapoda zoea comprises several stages of zoea including protozoa and mysis. In contrast, in Brachyura the protozoa and mysis stages are missing. The zoea phase normally presents 4 to 6 stages which identified based on molting in marine and estuary crab (Anger, 2001). Brachyuran crab zoea has a typical dorsal spine, and a rostral spine extending from the carapace. Morphologies of the zoea (Fig.2.8 B, C) can be distinguished from nauplius phase particularly in terms of change of function for thoracic appendages (Anger, 2001). Keys to identify planktonic species of crabs usually focus on the character of cephalothorax appendages: antennule, antenna, mandible, maxillule, maxilla, the three pairs of maxillipeds, and five pair of

pereiopods (Paula, 1996; Pessani et al., 1998). The pleonal or abdomenal appendages are still rudimentary and have less or no functions at that phase. Most of the cephalothorax appendages are involved in the locomotory function (swimming).

In some species and groups of Brachyura, the number of zoea stage can be reduced, in that case, it is started that the development is an abbreviated (Anger, 2001). This abbreviation can be more or less pronounced depending on groups. For instance, 13 of the 126 species of the dromiidae family crab for which zoea development diverge ranges from 1 to 6 stages (McLay et al., 2001). Abbreviation of larval development in marine and estuary crab seems to be fairly common in stressful environmental conditions and when food resources are limited. These can be the case of polar waters, deep waters, and in some estuary conditions (Bauer, 2004). This abbreviation can also occur when the way of life of the crab becomes symbiotic or parasitic as in pea crabs who usually develop in their hosts (Bolaños et al., 2005).

After zoea phase the megalopa phase (Fig.2.8D) is the last larval stage in marine and estuary crab. It sometimes referred to a “postlarva” (Martin et al., 1985). The cephalothorax appendages are completely developed like a juvenile crab. The greater pair of claws is observable. The megalopa phase is usually benthic as in the juvenile. The abdominal unit of megalopa however unfolds under its cephalothorax and is used for swimming unlike juvenile crabs (Anger, 2001).

Direct development of freshwater crab

In true freshwater crab or primary freshwater crab, the development can be extremely abbreviated with no planktonic phase. This group release benthic juveniles or juvenile-like megalopa from the mother. This form of development is usually known as “direct development” (Pace et al., 1976; Anger, 2001; Yeo et al., 2008;

Cumberlidge et al., 2009; Wu et al., 2010). Freshwater crabs have a direct development in that the embryonic development inside the egg produce juvenile hatchling crabs without passing via larval stage outside the egg. The embryonic development in eggs can, however, allow to forms that are similar to the free-living larva of marine crabs: in that case, these stages are referred as egg-zoea, and egg-megalopa to correspond to the free-living zoea and megalopa phase in marine crab. Freshwater crabs produce a smaller number of eggs, but with larger yolk eggs that remain attached to the abdomen of the mother during the achievement of their development until hatching. After hatching to be a juvenile, the little freshwater crabs are retained several days in a mother's abdominal pouch before to be released as free-living crabs (Liu and Li, 2000; Sant'Anna et al., 2013).

CHAPTER III

Study Area

General description

The study site is a rice field located at Lai Nan sub-district, Wiang Sa district, Nan province, northern part of Thailand (Fig 3.1; 47Q 0686779, UTM 2047187).

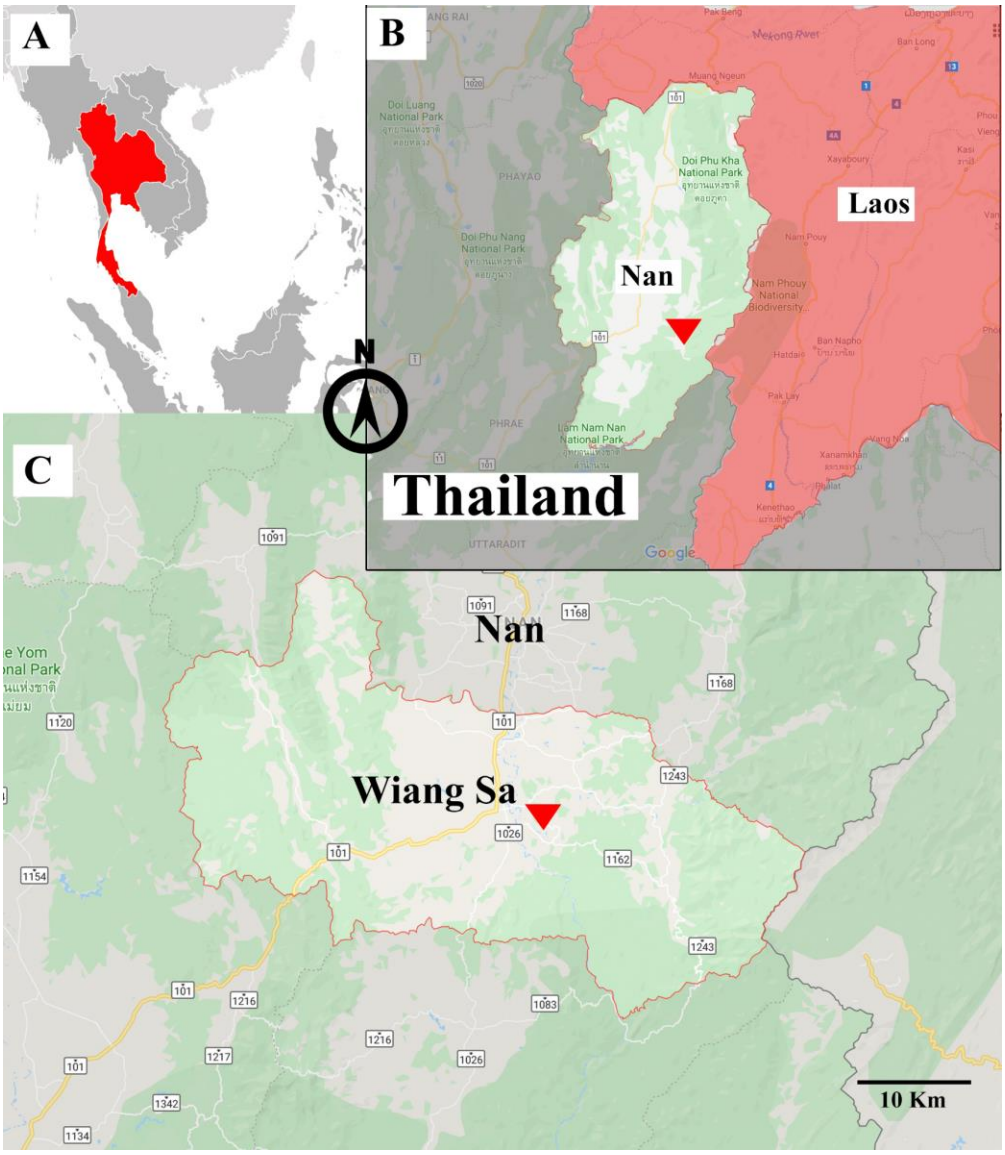


Figure 3.1 Map of the study site A) Nan province in the northern part of Thailand shown in red filled area; B) Wiang Sa district in Nan province indicated by red triangle mark; C) The study site in Lai Nan sub-district, Wiang Sa district indicated by red triangle mark. (GoogleMap®2019)

Figure 3.2 Satellite image of the study site in Lai Nan sub-district, Wiang Sa district, Nan province. The rice field used in this study is within the red rectangle. Blue solid arrow represents an irrigation canal with inflow from Wa river to the rice field, while the blue dashed arrow represents a canal with outflow from the rice field to Nan river.

The study area locates in Nan river basin between the Phi Pan Nam Range in Thailand and the Luang Prabang Range in Thailand and Laos. Wa river is one of tributaries from the Luang Prabang Range that flow into Nan river, a major river of Nan plain, at Wiang Sa district. With Nan river flows downward and further away from Lai Nan sub-district, Wa river become the major water resource for most of local agricultural activity in Lai Nan sub-district. Similar to other areas in the northern part of Thailand, water availability is relatively low for plantation in the plains between the mountains. The water level in the rice field is lower than 30 centimeters which is much lower than overall water level in the rice field of central Thailand.

Agricultural activity

The study field is regarded as an intensive rice farming (Fig. 3.3). Based on the Rice Intensification System (RIS), the intensive agriculture is a technique designed to increase the yield of rice production. The method uses intensive labors for planting single or small group of rice seedlings and weed management. It requires the plant nursery prior to transplanting to the large rice field. This method enables area with low water availability to gain more yield (Hameed et al., 2011). In addition, This particular rice field is also known as an agricultural field with no history of agrochemicals application for more than 10 years (Jantawongsri et al., 2015)

With the limited water availability, only one cycle of rice cultivation in each year can be carried out in this area. Rice cultivation usually starts at the beginning of rainy or wet season every year when the water from Wa river flows into the study site through the local irrigation canal (Fig 3.2). Rice cultivation begins once the rice field is filled with water and becomes an artificial wetland. There are several varieties of rice and sticky rice planting in this rice field (Fig. 3.3). However, crop cycle

(approximately 90 days) is relatively similar among varieties of rice that grow in this area. After harvesting, the rice field is usually free from other activity in the dry period. This allows soil to expose to sunlight and undergo natural restoration process every year. Hence, minimal number of weeds is found in the next crop cycle.

Figure 3.3 Intensive rice farming with no use of agrochemicals at the study site in Lai Nan sub-district, Wiang Sa district, Nan province. The sticky rice (variety Nan 59) nursery is shown in the front, while seedling transplantation is shown in the background.

Rice cultivation in this area largely depends on rainfall in wet reason as well as water supply from irrigation system. To ensure the availability of water, the rice field in this area is always supplied with water in the irrigation canal. This results in the constant water level in the rice field during crop cycle. The stable water resource is not only useful for rice, but also creates an ephemeral aquatic environment in the artificial wetland for other plants and animals.

Climate

The crab sampling period in this study started from November 2015 to November 2016. Within this period, seasons were classified based on climograph of plots between average temperature (degree celsius) and rainfall (mm) of Nan province recorded by the Thai Meteorological Department (Fig. 3.4). The seasons were classified into dry and wet seasons based on the fact that the wet season usually gets rainfall (mm) at twice as much of the average temperature in degree celsius (Walter et al. (1975). As a result, the period with rainfall above the average temperature line represents the potential wet season in this area. Also, since the tropical summer zone such as northern Thailand usually has an average rainfall of 100 mm per month during wet season, this amount of rainfall (100 mm) were used as a secondary criterion for delimiting the wet season. Any period that did not meet the former 2 requirements of the wet season would be regarded as the dry season. Therefore, local climate during this sampling period (November 2015 to November 2016) was listed as follows:

1. November 2015- January 2016: an early dry period (Fig. 3.5A)
2. February 2016- April 2016: a late dry period (Fig 3.5B)
3. May 2016- June 2016: an early wet period (Fig 3.5C)
4. July 2016-September 2016: a late wet period (Fig 3.5D)
5. October 2016- November-2016: an early dry period (Fig 3.5E, F)

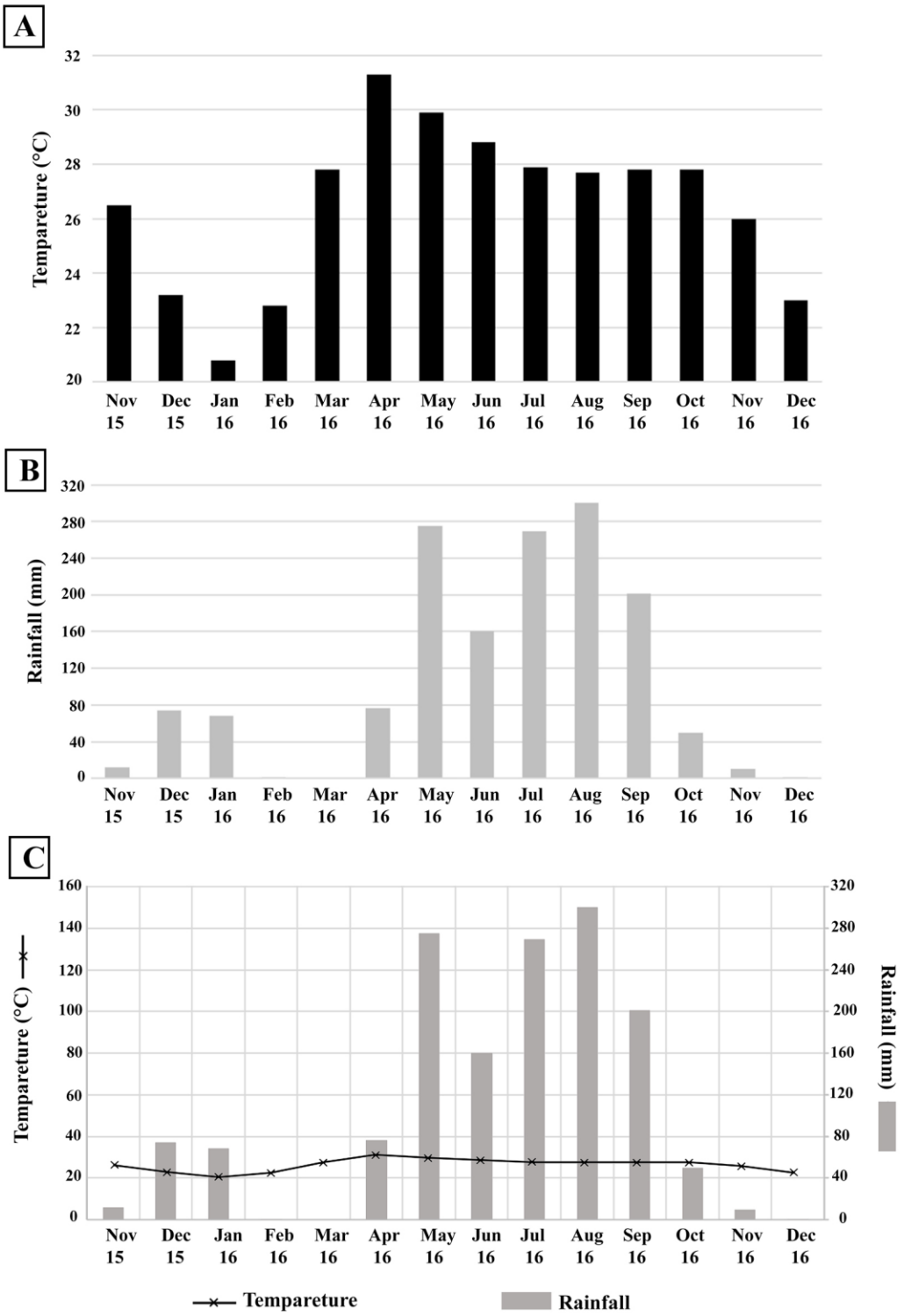


Figure 3.4 A) Average temperature; B) Average rainfall; C) Climate diagram (climograph) of Nan Province during the sampling period (November 2015 to November 2016). The period that has an average rainfall higher than twice the value of an average temperature is regarded as the potential wet season. These data were recorded by the Thai Meteorological Department.

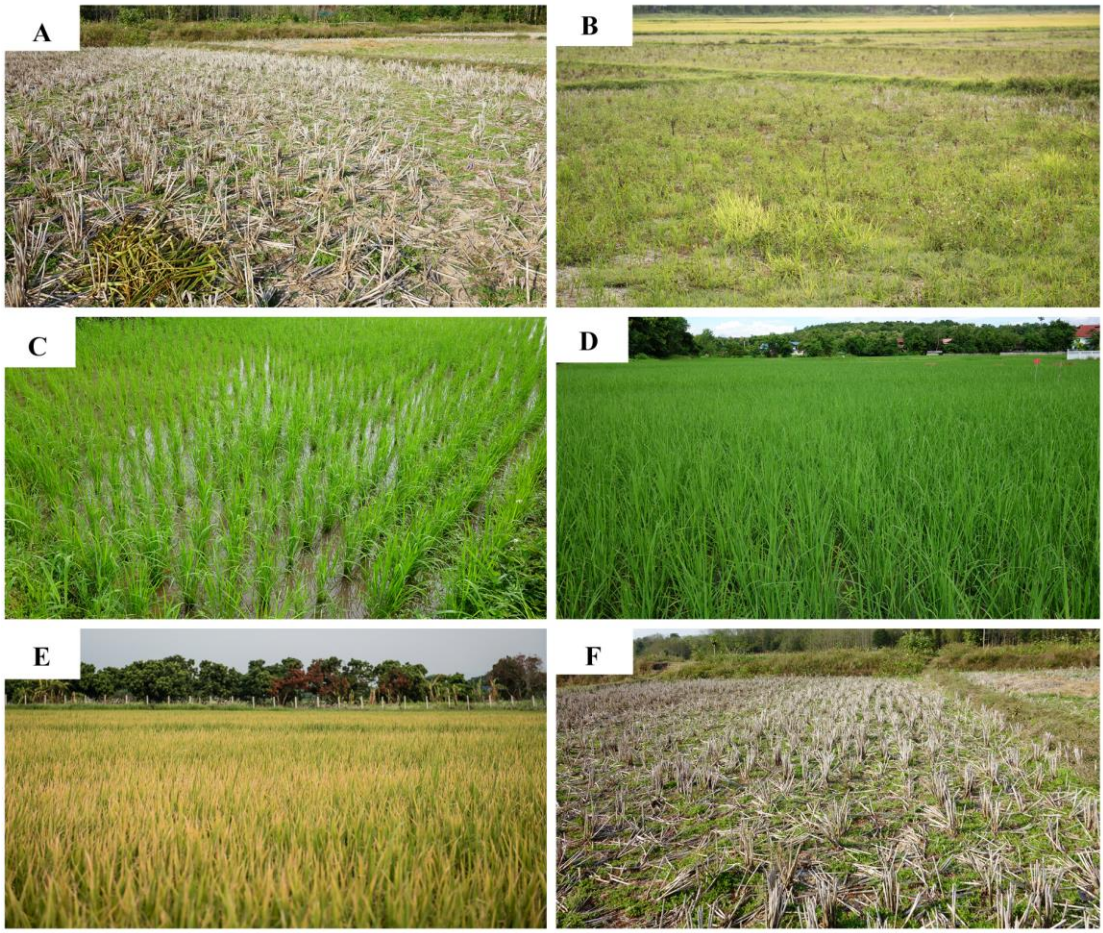


Figure 3.5 The representative pictures of rice field habitat at different seasons at the study site in Lai Nan sub-district, Wiang Sa district, Nan province: A) field after cropping season in an early dry period of 2015; B) field filled with grass in a late dry period of 2016; C) intensive rice cultivation in early wet period of 2016; D) field with 2-month old rice in late wet period of 2016; E) field with rice that was ready to harvest in an early dry period of 2016; F) field after cropping season in an early dry period of 2016.

Rice field crab <u>Esanthelphusa</u> <u>nani</u> (Naiyanetr, 1984)

Esanthelphusa nani was firstly described based on specimens caught at Nan Province (Naiyanetr, 1984) (Fig 3.6). Its classification is as follows:

Kingdom Animalia

Phylum Arthropoda

Subphylum Crustacea

Class Crustacean

Superorder Brachyura

Order Decapoda

Family Gecarcinucidae

Genus *Esanthelphusa*

Species *Esanthelphusa nani (*Naiyanetr, 1984*)*

Figure 3.6 Male rice field crab *Esanthelphusa nani* (Naiyanetr, 1984)

E. nani carapace is quite smooth and broad with epigastric cristae and separated by Y-shaped groove. There are 4 epibranchial separating from epigastric cristae tooth which is a main characteristic of *Parathelphusa*. Postorbital cristae end

beyond outer edge of distinctly sharp postorbital cristae like a H-shaped depression. Third maxilliped with flagellum is longer than width of merus. Strongly asymmetrical characteristics of chelipeds (major claw and minor claw) is occurred in larger males. There is a strong sharp carpus in the inner distal spine of the chelipeds. There is a T-shaped segment 7 with lateral margins slightly concave (neck-like appearance) in male abdomen.

Figure 3.7 Comparison of male and female rice field crab, *E. nani*, morphology. A) dorsal view of male crab. B) dorsal view of female crab C) ventral view of male crab. D) ventral view of female crab.

In general, male and female *E. nani* exhibits several sexually dimorphic characters. The sexual difference consists of size and shape of abdomen and claw size (Fig. 3.7). Male and female crabs similarly have six segments of abdomen plus one telson, but the abdomen of the female crab is broader than those of the male crab. In

the male crab, claw size shows a bilateral asymmetry with one larger claw (major claw) on one side and one smaller claw (minor claw) on another side. On the other hand, the claw size in female shows a bilateral symmetry with fairly similar size both claws.

The rice field crab inhabits mainly in the rice field and potentially adjusts their life cycle to be synchronized with local climate and agricultural activities. Its foraging season strongly associates to rice cultivation period. Since the crab is a semi-aquatic animal, the water body in the rice filed is critically needed for vital activities of crabs such as feeding, breathing, molting, burrowing as well as courtship and mating. When the rice field is void of water, the crab starts to deposit an essential nutrient in their hepatopancreas and be ready for estivation. The estivation of the crab lasts for a full dry season. Reducing activity and locomotion are the keys for saving its energy. Hiding in its burrow which is approximately 70 cm depth allows the crab to prevent the moisture loss during dry period. It is of important to note that some female crab invests high amount of energy for producing eggs and the hatchlings in the late dry season. This could be beneficial to increase fitness or competitive advantages of their offspring when the water is filled up in the rice field in the upcoming wet season.

CHAPTER IV

Anatomy and Histology of Male Reproductive Organs of the Rice Field Crab *Esanthelphusa nani* (Naiyanetr, 1984)

INTRODUCTION

Infraorder Brachyura (true crabs) is a major group of decapod crustaceans with an enormous number up to 7,000 species. Ecology of this group is diverse with various habitats ranging from marine, estuary, and freshwater environments. Crabs have adapted to these three environments by using different strategies. Marine crabs are exclusively marine, secondary freshwater crabs live in freshwater or estuarine habitat but must come back to the sea to achieve their reproduction, and primary freshwater crabs live in freshwater and estuarine environment from their larval to adult stages (Ng et al., 2008; De Grave et al., 2009). In terms of reproduction, the whole group has evolved a male reproductive organs to ensure reproduction. The gonopore is indeed transformed in a penis-like organ and its presence has been used to differentiate eubrachyurans (true crabs) from primitive crab-like Podotremata (Guinot, 1979). On the other hand, the internal reproductive organ of Brachyurans is similar between primitive crabs and evolved ones. (Castilho et al., 2008; Erkan et al., 2009; Stewart et al., 2010).

Male reproductive tract of crabs consists of two major different regions: testis and vas deferens (Sainte-Marie and Sainte-Marie, 1999). The testis normally locates anterodorsally in the cephalothorax. The testis consists of coiled and convoluted seminiferous tubules where the spermatogenesis occurs (Stewart et al., 2010; Zara et al., 2012). At the end of seminiferous tubules, the vas deferens transfer the sperm into

to the penis like organ. The vas deferens can be divided into three parts: anterior, middle, and posterior (Johnson, 1980; Krol et al., 1992). The sperm is usually packed and enclosed in several spermatophores. In some species, the sperm packing into spermatophore occurs in the anterior vas deferens (Sainte-Marie and Sainte-Marie, 1999; Zara et al., 2012), but some species are reported to show the spermatophore in the middle vas deferens (Soundarapandian et al., 2014). The role of these variations has not yet been well defined. Moreover, male reproductive tract microstructure can also show variation, but the function of these has rarely been documented.

In general, the amount and quality of the sperm and other ejaculated components that the male subsidizes affect directly to the ability of females to fertilize their eggs in most animal species (Keller and Reeve, 1995; Reynolds, 1996). Several factors can act on sperm quantity and quality (Sato and Goshima, 2007; Nagaraju and Borst, 2008; Harlıoğlu et al., 2018). In crabs, the amount of male gametes released by male and reaching females may be low depending on the mating frequency of males, since they might require time to reconstitute the stock of gametes and seminal fluid (Sato et al., 2010). The amount of sperm can vary along the year and display the seasonal changes (Sato et al., 2010). Environmental factors such as temperature, salinity, light periods could influence on testicular growth in crabs (Hunter and Naylor, 1993; Warman et al., 1993) and these factors can also show seasonal variation.

While a few studies have addressed change in male reproductive organs and histology in marine crabs, *Maja brachydactyla* (Majidae), *Portunus pelagicus* (Portunidae) (Simeó et al., 2009; Zara et al., 2012; Soundarapandian et al., 2014) and secondary freshwater crabs, *Goniopsis cruentata* (Grapsidae), *Ucides cordatus*

(Ucididae) (Garcia and Silva, 2006; Castilho et al., 2008), few studies have been done on male reproductive biology in freshwater crabs. This work presents a study documenting the histology and seasonal change in the gonads of the crab *Esanthelphusa nani*, a member of the Gecarcinucidae which is a family comprising only primary freshwater crabs. It was aimed to reveal gonad histology and morphology in Gecarcinucidae and determine whether histology and morphology can display change due to seasonal variation. In particular, and because this crab is living in Northern Thailand characterized by a dry and rainy season, it is hypothesized that sperm production might be affected by the availability of resources during the year. Since the seasonal variation was found in females (see Chapter V), it was aimed to determine whether seasonal changes of the male are in synchrony or not, so that the reproductive biology of the crab can be discussed.

MATERIALS AND METHODS

Crab sampling

The study site is described in Chapter III. A field survey was carried out from dusk to late night with a visual encounter survey approach. The sampling was carried out between November 2015 and November 2016. Five adult male crabs with a carapace width of more than 30 mm were hand caught on a monthly basis. The crabs were then transported to a local laboratory for one-night acclimatization. Then, the crabs were later euthanized using the cold shocking in ice slurry. Measurements including weight and carapace width were taken in order to eventually correct for size/weight.

Male reproductive tracts examination

Macroscopic examination

The carapaces of male crabs were cut off. The heart and digestive tract were eliminated carefully in order to present the male reproductive tract. Then, the male reproductive tract (above the hepatopancreas) was photographed in the cephalothorax cavity with a Nikon d750 camera and an AF-d 60 mm lens to deliver a fine detail picture. The color of white balance was calibrated and set to all pictures by using a standard 18% grey card in order to get a consistence exposure of color tone in any given picture. The picture of reproductive tract was observed, described and used as a descriptive source of information. The reproductive tract was also described based on its size in the cephalothorax cavity. The whole male reproductive tract was gently removed out of the cephalothorax cavity and subject to gravimetric study. Gonadosomatic index (GSI) was calculated as the formula:

GSI = [the weight of testes and vas deferens /total weight of crab] × 100

Finally, the whole male reproductive tract was fixed in Davidson's solution overnight. Tissues were subsequently preserved in 70% ethanol solution before histological procedure for microscopic examination.

Microscopic examination

The whole male reproductive tract was observed in 3 individuals in terms of histology for checking the homogeneity of the testis and vas deferens (VDs). As a preliminary result, the testis tissue showed a fairly homogenous tissue (anterior and posterior testis of left and right sides). Then only left middle testis (N = 3) for every month were selected as representatives for microscopic examination of the testicular

cycle. Differences in tissue structure were found along the vas deferens (VDs). Therefore, the VDs were dissected into anterior, middle, and posterior parts based on locality of VDs. The number of representative VDs section for microscopic examination were therefore 3 per individual and per month. As for testis, three individuals were observed every month. The testis and VDs were embedded in paraffin using the following method: they were first dehydrated by immersion in a graded alcohol series, then cleared in xylene, and finally embedded into blocks. Sections at 6 μM were suitable for observing testicular and VDs tissues. The testicular section and the three VDs sections were stained by hematoxylin and eosin (Appendix B). In addition, the Periodic acid–Schiff staining method (PAS: Appendix B) was used because of its specificity to carbohydrate expressed in basement membrane and allowing boundary of seminiferous tubules in testis to be recognized. All tissues were explored under light microscope and photographed by Canon 7DII camera.

Data analysis

The histological and morphological structure of testis and VDs were descriptively documented for basic information to study testicular cycle. The normality of monthly GSI was estimated using the test of Shapiro–Wilk prior to further statistical comparisons among months. One-way ANOVA with the month as explanatory factor, followed by the Tukey post-hoc test, was used to compare GSI. Annual changes and stage in male reproductive cycle were based on significant difference of the GSI among month. Microscopic and macroscopic differences in testis and VDs were compared morphologically among the different stages found in the cycle.

RESULTS

General descriptive appearance of male reproductive organ of E. nani

The male reproductive organ of *E. nani* presents in an 'H' shape. The tract consists of a pair of testis and vas deferens (Fi. 4.1). The reproductive tract has a creamy to white color. The paired testes occupy the expected positions in the anterior cephalothorax, above the hepatopancreas. The left and right sides of the testis are connected by a commissure bridge located at the posterior part of the testis. The left and right VDs are found at the posterior end of the respective testis, just after the commissural bridge. The testis can be distinguished from VDs based on texture and color. The color of testis is slightly more yellowish than the color of VD. The three different regions of the VDs can be described according to antero-posterior plane of cephalothorax. The anterior vas deferens (AVD) locates next to the posterior testis near the commissure bridge. AVD shape corresponds to a thin tubule that is highly convoluted. Middle vas deferens (MVD) locates after AVD construction and has a larger size. The posterior vas deferens (PVD) is a narrower duct that locate after the MVD. The appearance of PVD is less convoluted that the appearance of AVD. The rear part of PVD opens at the ventral coxa of the fifth walking leg.

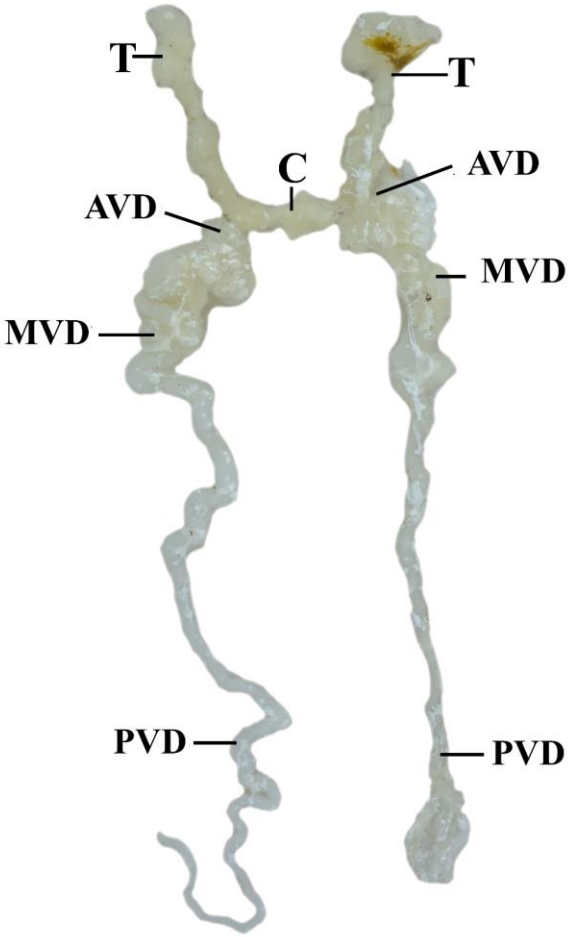


Figure 4.1 Dissection of the internal reproductive tract of male *E. nani* [representative of testis in June 2016]. It shows an 'H' shape which consists of testis and vas deferens. AVD: anterior vas deferens; C: commissure bridge; MVD: middle vas deferens; PVD: posterior vas deferens; T: testis.

Microstructure of testis and seminiferous tubules of E. nani

The testis consists of numerous tubules containing convoluted seminiferous tubules (Fig. 4.2B). Each convoluted seminiferous tubule is enclosed by thin seminiferous epitheliums on basal lamina (Fig. 4.2A). Three zones in seminiferous tubules based on different germ cell activity can be defined from the periphery to the lumen: 1) germinal zone (GZ), 2) transitional zone (TZ), and 3) evacuated zone (EZ) (Fig. 4.2D).

Histological results show that sections of seminiferous tubules are at different stages of the spermatogenesis, which form a spatial and temporal spermatogenetic wave (Fig. 4.2A) from the periphery to the lumen and along the seminiferous tubules.

The germinal zone (GZ) is at the periphery of the seminiferous tubules. Spermatogonia can be found in the germinal zone at the level of the prominent seminiferous epithelium or germinal epithelium (Fig. 4.2C, Fig. 4.3A-C). Nurse cells presenting an un-oval and a typical small nucleus can be found. The germinal zones exhibit along seminiferous tubule (green arrow in Fig. 4.2A) that refer to the long-section model of Fig. 4.2D. Mitotic division allows spermatogonia to proliferate in the germ zone. Some of the daughter cells keep their proliferative role for ensuring the spermatogonia stock, whereas the other part differentiates into primary spermatocyte. The primary spermatocytes subsequently proceed the meiosis division in the direction of the seminiferous lumens in a zone that is called transitional zone.

In the transitional zone (TZ), spermatogenesis and spermiogenesis progress and different germ cell stages can be found, together with seminal fluid (Fig. 4.2C, D). Primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa can be found in that transitional zone.

TZ is a zone at lumen site of the seminiferous tubule. The zones consist of different germ cell stages and seminal fluid. The germ cells are at different spermatogenesis and spermiogenesis stages throughout the seminiferous tubule (Fig. 4.2C, D). Histological results show different colors of seminiferous lumens which exhibit different spermatogenesis and spermiogenesis (Fig. 4.2A). There are primary spermatocytes, secondary spermatocytes spermatids, and spermatozoa in the transitional zone. Since the spermatogonia have an interval time of the spermatogenic

cycle in each GZ. The same interval time lead to the presence of the same stage of spermatogenesis in TZ. There is a hypothesis that the activator message of the germ cells in the TZ spread along seminiferous tubules to communicate with spermatogonia of each GZ. The result of communication of GZ present to synchronize germ stage in a wave like appearance in the TZ, or nominally called the spermatogenic wave. The end of transitional zone connected to evacuated zone.

Evacuated zone (EZ) is a zone where products of the spermatogenesis (spermatozoa) are evacuated in direction of the anterior VDs. The sections showing that the zone is narrow, which means that seminiferous tubules in EZ are narrow and compressed by the surrounding seminiferous tubules in TZ. In the section, this zone has a dark blue color due to hematoxylin staining.

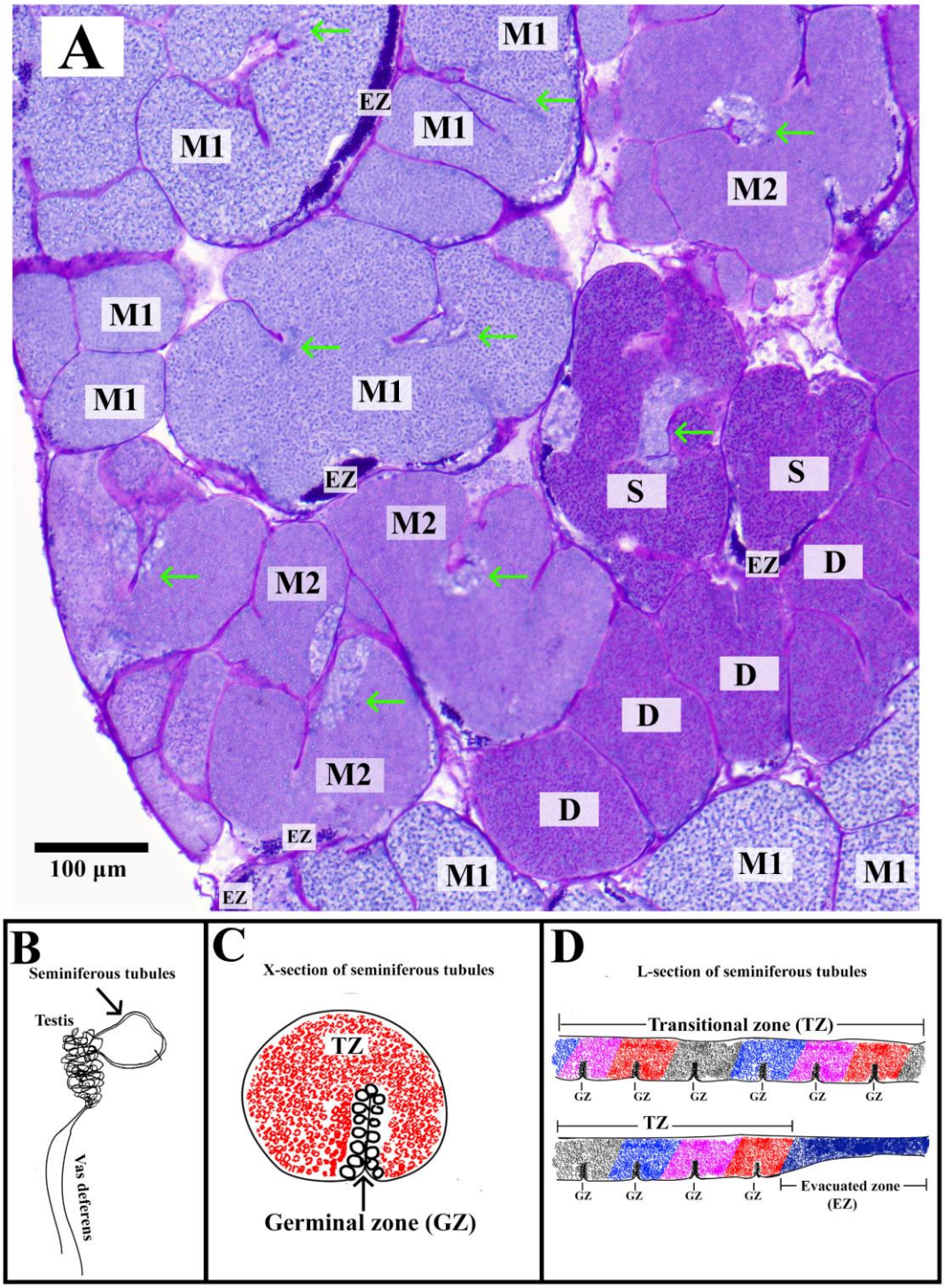
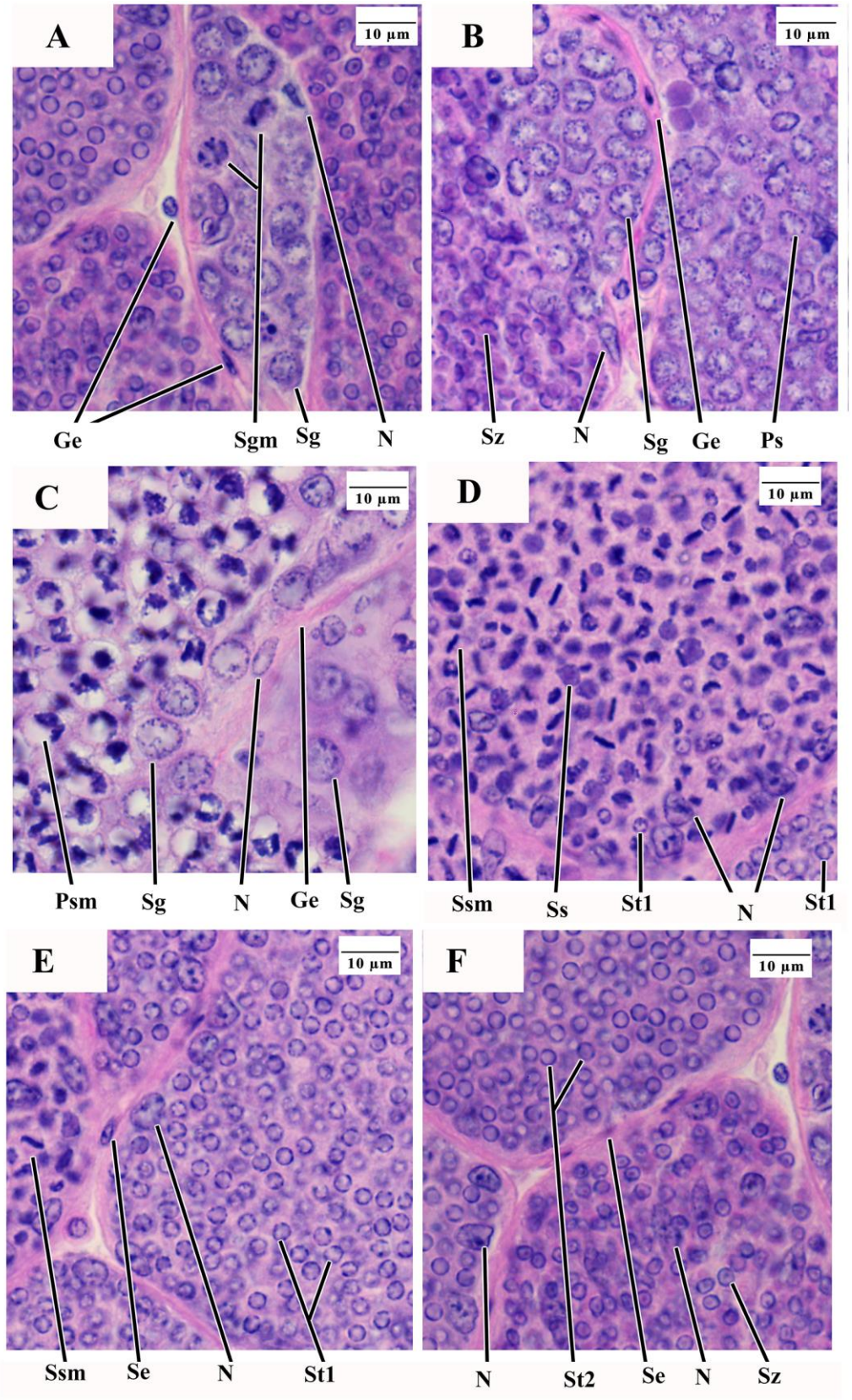


Figure 4.2 Overview testis structure and presenting model of testis (explanation shows in the next pages).

Figure 4.2 Overview testis structure and presenting model of testis. A) Section of testis showing seminiferous tubule structure. The seminiferous tubule shows the convoluted tube structure. The different colors in seminiferous tubule present different stages of spermatogenesis and spermiogenesis. Spermatogonia (green arrows) present on the germinal epithelium that is elongated into the seminiferous tubule (PAS stain); B) Interpretative drawing of testis and seminiferous tubule; C) X-section showing the germinal zone in black color (black arrow) and the transitional zone in red color; D) L-section of drawing model of seminiferous tubule. The model shows three different zones along the seminiferous tubules. The Germinal zone presents the spermatogonia (black arrow) on the germinal epithelium. The transitional zone is the place for the spermatogenesis and spermiogenesis. The different colors show groups of cells that are in the same cycle of spermatogenesis and spermiogenesis. The evacuated zone presents dense spermatozoa which go in direction of the lumen of seminiferous tubules toward the vas deferens. D: differentiation of spermatids in TZ; EZ: evacuated zone; GZ: germinal zone; M1: meiosis I of spermatocyte in TZ; M2: meiosis II of spermatocyte in TZ; TZ: transitional zone; S: spermatozoa in TZ.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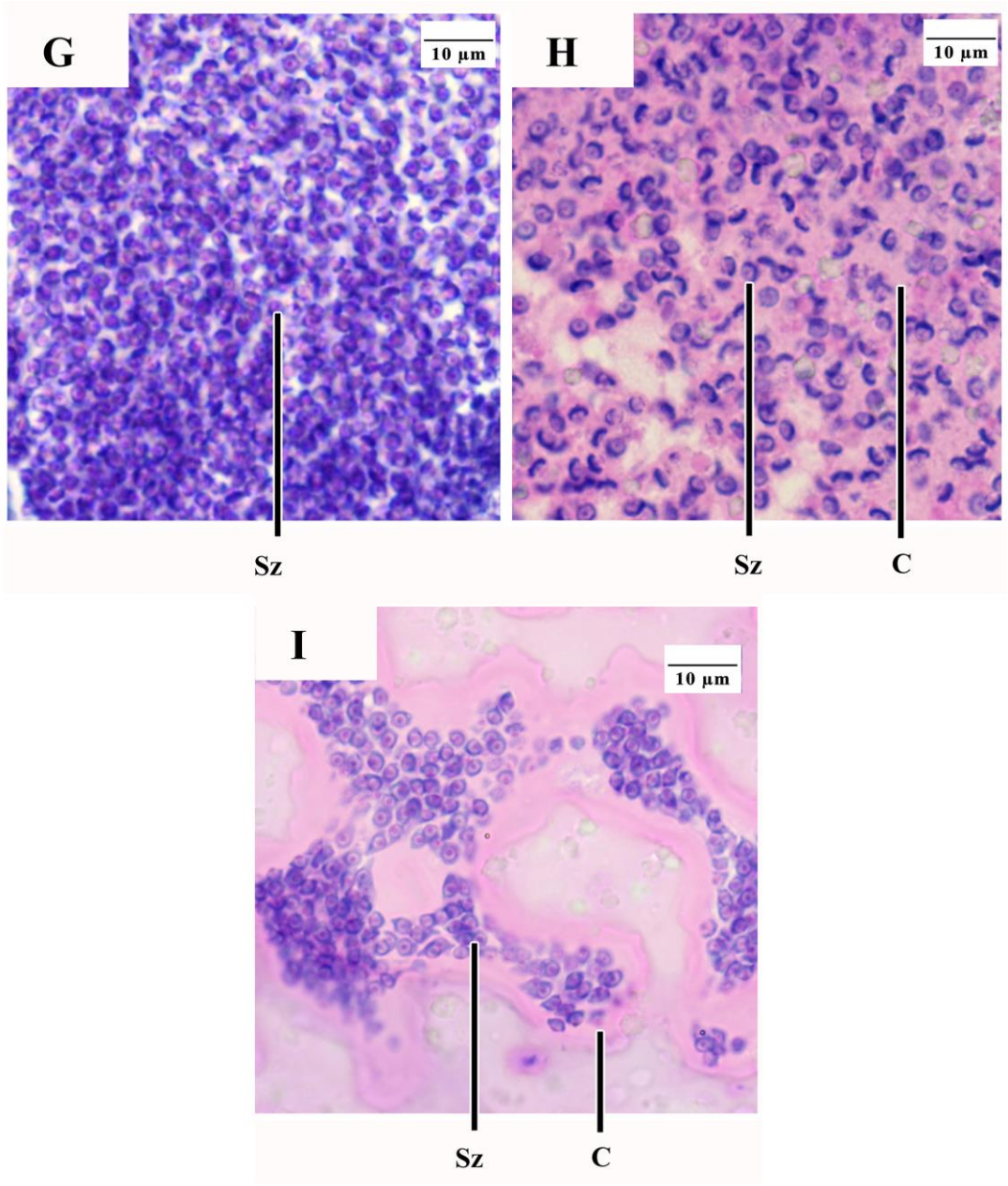


Figure 4.3 Section of seminiferous tubules shows spermatogenesis and spermiogenesis. A) The germinal zone in resting phase of spermatogonia (Sg); A group of spermatogonia presents on the germinal epithelium; B) The germinal zone in active phase of spermatogonia. Spermatogonia process rapidly on cell division (Sgm) and differentiate to be primary spermatocyte (Ps) far away from germinal epithelium; C) Primary spermatocytes are at meiosis I (Psm) in transitional zone that district from germinal zone of spermatogonia; D) Secondary spermatocytes are at meiosis II (Ssm) in transitional zone. Several spermatids (St1) are present in transitional zone; E) Spermatid I (St1); F) spermatid II (St2) and spermatozoa (Sz) in transitional zone; G) Spermatozoa present in evacuated zone of seminiferous tubules; H) vas deferens; and I) spermatheca of female crab.

Spermatogenesis and spermiogenesis in <u>E. nani</u>

This study classified germ cells within the seminiferous tubule into 5 stages based on cytological characteristics (Fig. 4.3). There are spermatogonia, primary spermatocytes, secondary spermatocyte, spermatids, and mature sperm.

Spermatogonia (Fig. 4.3A-C)

Clusters of spermatogonia are found along the fold of the germinal epithelium. There are two forms of spermatogonia. The first form is large with evenly dispersed heterochromatin (diameter: 7.6 ± 0.8 μm; *N* = 40 cells). This form is commonly known as the resting stage. The second form is the spermatogonia at mitotic division (diameter: 5.4 ± 0.9 μm; *N* = 40 cells). Cells are large and the nuclear membrane cannot be seen. The chromatin is condensed. The daughter cells of those mitotic spermatogonia usually exhibit smaller cell (diameter: 5.2 ± 1.9 μm; *N* = 40 cells). Some daughter cells eventually differentiate into primary spermatocyte.

Primary spermatocytes (Fig. 4.3C)

Primary spermatocytes (Ps) are first similar to spermatogonia. Ps spend time in interphase to grow to about 6.3 ± 0.6 μm in diameter (*N* = 40 cells). Then, the mature Ps process the meiosis I. At meiosis I, the cells are round with condensed chromatin near the metaphase plate. The cytoplasm of Ps is large with shade the seminal fluid in the background. At this phase, the Ps can be well differentiated from spermatogonia in the germinal epithelium.

Secondary spermatocyte (Fig. 4.3D)

After meiosis I, the two sister cells of Ps become secondary spermatocytes (Ss). In the study, Ss were difficult to observe because the interphase is absent or very short. Indeed, most of the Ss were found in the meiosis II. Since the Ss lack of the interphase, their size (diameter: 4.0 ± 0.3 μm; *N* = 40 cells) are smaller than those of Ps. The small amount of Ss is uncovered the color of seminal fluid background that present by eosinophilic color. The chromatin aspect depends on the nuclear division phase: prophase II, metaphase II, and anaphase II.

Spermatids (Fig. 4.3E)

Upon the completion of Meiosis II, spermatocytes produce the spermatids (St). This study recognizes two forms of St. The two forms are about the same size (diameter: 2.7 ± 0.3 μm; *N* = 40 cells), but differ in terms of chromatin condensation. The St1 retains uniformly lightly condensed chromatin which is a general form of early phase. The St2 forms are more advanced than those form in St1, and the chromatin is more condensed as a result of spermiogenesis.

Mature sperm (Fig. 4.3F-I)

Mature spermatozoa (Sz) is the final product presents at the end of spermiogenesis. Sz has completely condensed chromatin. A subacrosomal space or perforatorium is present as a small dot in the center of Sz. As in other crabs, the spermatozoa of *E. nani* do not have tail (Fig. 4.3F). Spermatozoa in the evacuated zone (Fig. 4.3G) or in vas deferens (Fig. 4.3H) are indistinguishable under light microscopy. Their appearance is also the same when they are stored in the female spermatheca (Fig. 4.3I).

Microstructure of vas deferens

Histological studies of the vas deferens (VDs) shows the tubular structure, which consists of a single layer or simple epithelium forming the wall that encloses the seminal fluid with spermatozoa (Fig. 4.4). Difference in the epithelium can be recognized and used to classify the VDs into anterior, middle and posterior VDs.

Seminiferous ducts open at the anterior vas deferens (AVD). The macroscopic structure shows thin and convoluted ducts. The microscopic structure shows the simple cuboidal epithelium forming the AVD ducts (Fig 4.4A, B). Circular muscle cells cover a single layer of connective tissue located at the basal laminar side of the epithelium (Ro et al., 1990). The lumen of AVD is narrow with dense spermatozoa (diameter: 50 ± 200 μm; N = 3 crabs).

The middle vas deferens (MVD) is the largest ducts. The MVD shows a simple squamous epithelium forming the ducts (Fig. 4.4C, D). Circular muscle cells are found at the basal laminar side of the epithelium. The lumen exhibits wide space where spermatozoa and seminal fluid are accumulating (diameter: up to 1500 μm in active testis crab). This part of VDs is highly associated to the testicular activity since the size of MVD depends on sperm accumulate in MVD lumen.

The posterior reproductive tract consists of straight and thin ducts classified as posterior vas deferens (PVD) based on macrostructure. The microscopic exanimation of PVD clearly shows a different histological structure by comparison to MVD. The epithelium that forms PVD duct is simple columnar epithelial cell (Fig. 4.4E, F). This epithelium is encased by two layers of muscular tissues which are internally circular muscle and externally longitudinal muscles. The whole duct structure is covered by connective tissue. The lumen shows small amount concentrated seminal fluid with

spermatozoa (diameter: up to 200 μm; *N* = 3 crabs). The presence of muscular tissue might be involved with the ejaculatory function of that part of vas deferens.

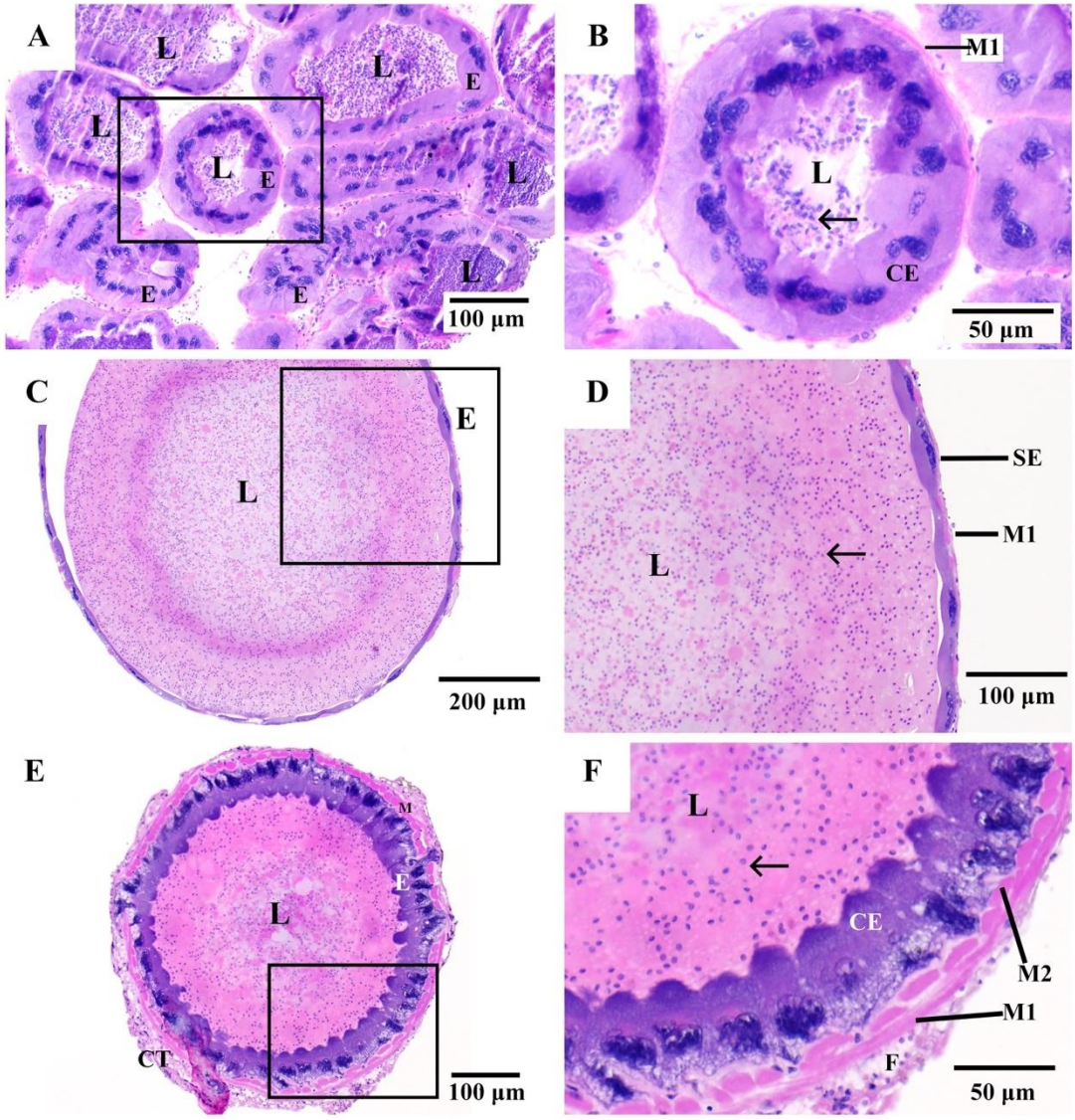


Figure 4.4 Section of vas deferens shows three types according to location. Cell structure and lumen size of the vas deferens are different. The vas deferens are able to categorize based on different epithelium types. A-B) anterior vas deferens formed by simple cuboidal epithelium; C-D) Middle vas deferens formed by simple squamous epithelium; E-F) Posterior vas deferens formed by simple columnar epithelium which surrounded muscular tissue. The arrow present spermatozoa and seminal fluid in the lumen. Cl: simple columnar epithelium; CT: connective tissue; Cu: simple cuboidal epithelium M1: circular muscle M2: longitudinal muscle L: Se: simple squamous epithelium.

Gonadal cycle based on gonadosomatic index (GSI) and testicular stage (Fig. 4.5)

Two seasons were characterized in the Northern part of Thailand's as shown by on the climograph obtained from the Thai Meteorological Department data (Chapter III). The GSI study shows that there were monthly changes of GSI in the male freshwater crab, *E. nani* (Fig 4.5A), and these changes were significant (ANOVA, $p < 0.05$). The GSI increased at the beginning of the wet season in May. The GSI levels were significantly higher in June and August (Tukey post hoc test, $p < 0.05$) and at maximum in September (Tukey post hoc test, $p < 0.05$). Then, GSI dramatically decreased until October in the early dry season. The GSI stayed low during the dry season, with a local increased in December that was also related to an increase of rainfall. The GSI mean plotting with average rainfall shows very high relationships between male gonad and environment (Fig. 4.5B). The Pearson correlation test indicates strongly positive relationship ($r = 0.4301$, $p<0.05$).

The macroscopic examination of male reproductive tracts showed that it was difficult to classify its aspect as different stages in a cycle (Fig. 4.5C, E, G). The reproductive tract color is mostly similar and always show a white color. It is later tried to see whether different levels of GSI could be related to microscopic and histologic changes in testis (Fig. 4.5A).

Three different aspect can be described in each stage. Stage 1 (Fig. 4.5D) shows small amount spermatogenic activity in seminiferous tubules. The seminiferous tubules are smaller with lesser area of transitional zones. The evacuated zone can be easily seen due to lesser area of TZ. The stage is called resting testicular stage.

In Stage 2 (Fig. 4.5F), spermatogenesis is active as evidenced by the important transitional zone. The lumen of the seminiferous tubules is however slightly smaller

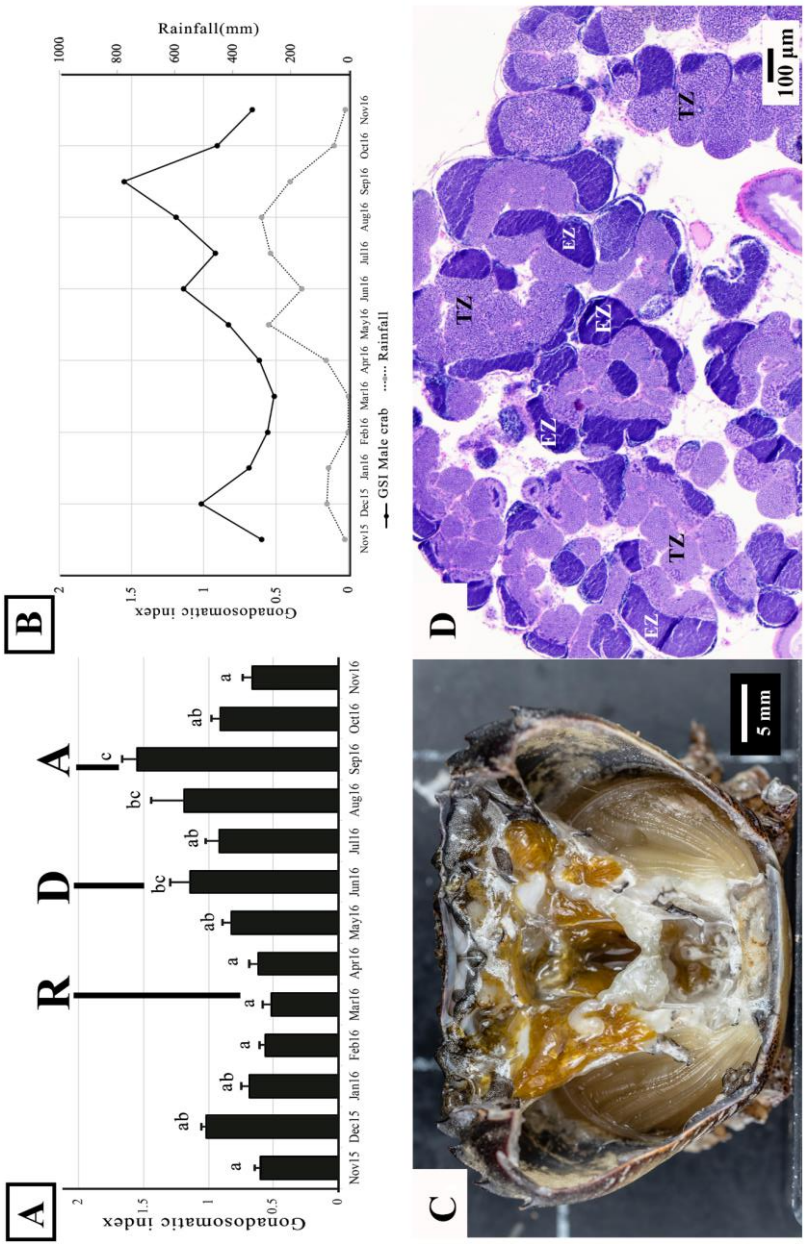
than those in the active testicular stage. An evacuated zone is present but compressed as in the active testicular stage. This stage is called a developing testicular stage.

Stage 3 (Fig. 4.5H) shows very active seminiferous tubules. Seminiferous lumens are large. The seminal fluid mainly exhibits the color of eosin stain. Evacuated zone is difficult to observe because the high testicular activity may compress this zone. This stage is called an active testicular stage.

Microscopic examination of vas deferens structure could not allow any important association with the different GSI. The microstructure was similar whatever the GSI or testicular stage. However, the size of middle vas deferens was showing some changes, with an increase of size, when testis was more active. The summary of comparative characters of male reproductive structure is shown in Table 4.1.

Table 4.1 Comparison of presenting appearance of zone of testis and secretion in vas deferens among active, developing and resting testicular stage. The plus symbol (+) indicates the degree of exhibiting.

Appearance / Stage of testis	Presenting zone of testis			Spermatozoa and secretion in vas deferens		
	Germinal	Transitional	Evacuated	Anterior	Middle	Posterior
Resting testis	+	+	+++	+	+	+
Developing testis	+	++	++	+	++	+
Active testis	+	+++	+	+	+++	+


A
B
C
D
Gonadosomatic index
Rainfall(mm)
GSI Male crab
Rainfall
Nov15 Dec15 Jan16 Feb16 Mar16 Apr16 May16 Jun16 Jul16 Aug16 Sep16 Oct16 Nov16
TZ
EZ
100 µm
5 mm

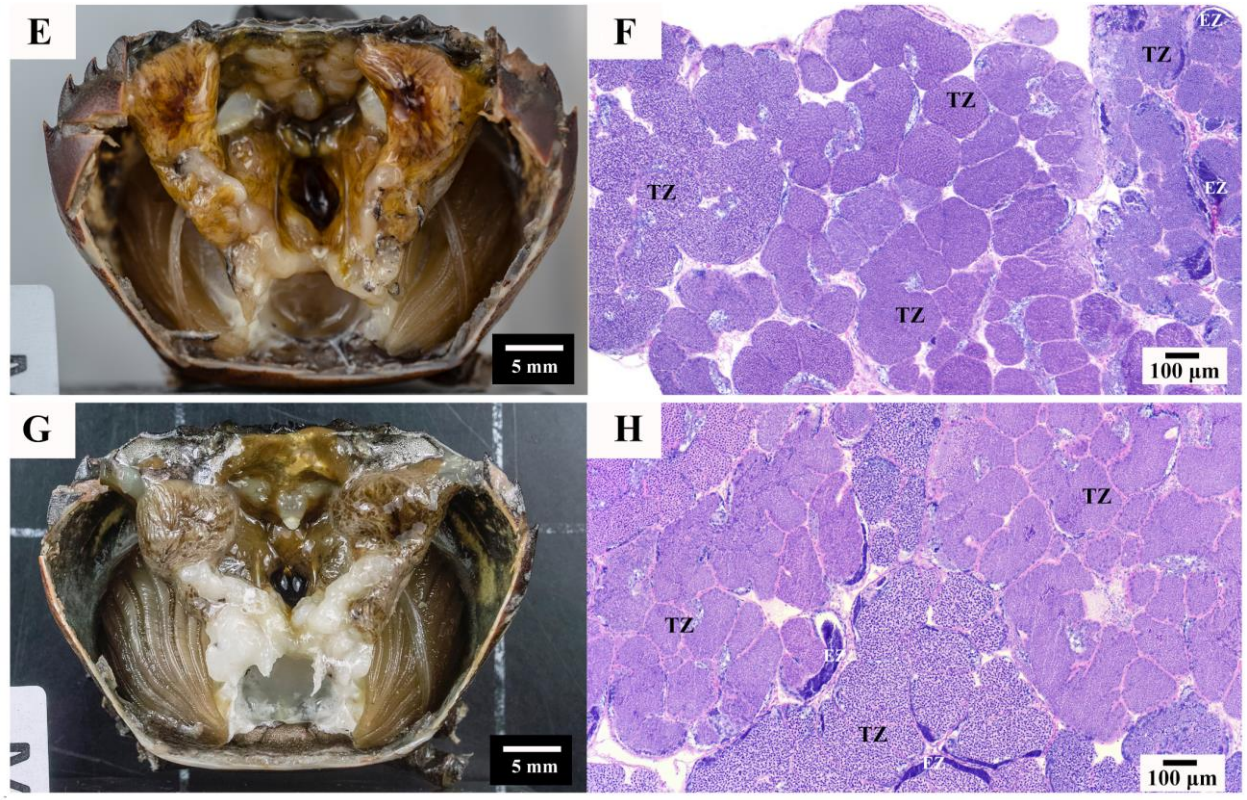


Figure 4.5 (A) Seasonal variation of gonadosomatic index of male rice field crab, *E. nani*, based on monthly samplings. The significant differences of GSI show in different alphabets (ANOVA, $p<0.05$). R, D, A alphabets are resting testis, developing testis, and active testis showing in Fig. C, E, G which are the representative of crabs in the March, June and August subsequently; B) Plotting means between GSI and the average rainfall shows significant relationship between testis activity and the rainfall factor (Pearson's correlation, $p<0.05$); C, E, G) Macroscopic examination shows overview of resting testis, developing, active stage; D, F, H) Microscopic examination show different testicular zone distributing in those testis stages. TZ: transitional zone; EZ: evacuated zone.

DISCUSSION & CONCLUSION

This study described the morphology of the gonad *E. nani*. It showed that it is similar to several described species such as *Maja brachydactyla* (Majidae, marine crabs), *Callinectes danae* (Portunidae, marine crabs), *Goniopsis cruentata* (Grapsidae, mangrove crabs), *Sylviocarcinus pictus* (Trichodactylidae, primary freshwater crab) and *Potamon fluviatile* (Potamidae, freshwater crabs) (Garcia and Silva, 2006; Simeó et al., 2009; Scalici et al., 2010; Silva et al., 2012; Zara et al., 2012). Cyst form explained in *G. cruentata* (Grapsidae, mangrove crabs) presented the same stages of germ encasing of squamous cells (Garcia and Silva, 2006). The lobular form is generally reported in crustacean species (Minagawa et al., 1994; Nagao and Munehara, 2003; Stewart et al., 2010; Zara et al., 2012). Simeó et al. (2009) proposed the tubular form of testis that consist of seminiferous tubule based on TEM of *M. brachydactyla* (Majidae, marine crabs) testis. The zones of seminiferous tubule have been used for explaining area of germ cell development including germinal, transitional, and evacuated zone (Simeó et al., 2009; Stewart et al., 2010; Zara et al., 2012). However, zones that exhibited repeatedly along seminiferous tubule has no intensive discussion. This study proposes the model of seminiferous tubule structure in the *E. nani* based on three zones. This model could be crucial to comprehend the seminiferous structure in testis in further study in Brachyura.

The epithelium of the VDs in *E. nani* can be classified based on functional and ultrastructural criteria. These criteria have been commonly used in other crab species (Castilho et al., 2008; Erkan et al., 2009; Stewart et al., 2010). However, differences exist among species and groups. Here is a review of what they are and try to see whether they can be related to phylogeny or ecology. AVD and MVD of *M.*

brachydactyla (Majidae, marine crabs) are composed of a single layer of epithelial cells with circular muscle cells (Erkan et al., 2009). The muscle contributes to spermatophore movement towards the MVD and PVD (Diesel, 1989). The epithelium of AVD usually presents as columnar or cuboidal cell types because they may have been involved in secretion (Sapelkin & Fedoseev, 1981.). The MVD thin cuboidal epithelium are commonly reported in *G. cruentata* (Grapsidae, mangrove crabs), *M. brachydactyla* (Majidae, marine crabs), and *C. danae* (Portunidae, marine crabs) (Garcia and Silva, 2006; Simeó et al., 2009; Zara et al., 2012). In the freshwater crab *P. fluviatile* (Potamidae, freshwater crabs), MVD is also organized as a thin layer (Scalici et al., 2010). In contrast, the MVD epithelium of *E. nani* is thinner than epithelium of AVD which was classified as squamous epithelium. As the major function of seminal storage, the main VDs weight is dominate by the MVD (Sainte-Marie and Sainte-Marie, 1999). The PVD is distinct from the two VDs. The single layer of epithelial cells that form VD duct covers with two muscle layers. The two muscle layers oriented in different directions (Ro et al., 1990). This structure function on transferring seminal component to female crab (Sudhadevi and Adiyodi, 1995; Erkan et al., 2009).

Sperm maturation, sperm packing, and seminal fluid production and storage all occurred in the crustacean VD of marine crabs (Hinsch and Walker, 1974). Occurrence of spermatophores is common in crabs and is usually formed from the AVD (Adiyodi and Anilkumar, 1988). Then, these spermatophores transit into MVD (Sainte-Marie and Sainte-Marie, 1999). The spermatophore wall may vary from group to group. In *M. brachydactyla* (Majidae, marine crabs), *Eriphia verrucose* (Eriphiidae, coastline crabs), and *Portunus pelagicus* (Portunidae, marine crabs), it is composed of

a single layer epithelium (Erkan et al., 2009; Simeó et al., 2009; Stewart et al., 2010). This structure also presents in *P. fluviatile* (Potamidae), and *S. pictus* (Trichodactylidae) freshwater crabs (Scalici et al., 2010; Silva et al., 2012). In this study, evidence of packing of spermatozoa in spermatophore in *E. nani* was not found in any portion of the vas deferens or even in the spermatheca of the female (Chapter V). The spermatozoa are directly in the seminal fluid. This absence of packing could be a unique character of the Gecarcinucidae family since it has been reported based on ultrastructure of sperm that the *Sundathelphusa philippina* (Gecarcinucidae) could lack of spermatophore formation (Klaus et al., 2013) as well.

Gonadosomatic index is a basic measurement to assess crab reproductive tract maturity and reproductive cycles. Immature, maturing, and mature stages are approximate identification based on GSI and macroscopic measurement including size and color of gonad (Silva et al., 2012). To exclude age variables, the adult must be selected necessary. As in some other crab species, this study shows a cycle in GSI and investment in reproduction. The seasonal changes refer to different variables including temperature, humidity, light period that could influence on the male gonadal development of crabs (Hunter and Naylor, 1993; Warman et al., 1993). *E. nani* presented the high relationship between GSI and rainfall. In freshwater crabs living with contrasted seasons, investment in reproduction may depend on availability of resources (food and mating pair, i.e. females). These can explain the seasonal pattern found in *E. nani* with reduced activity during the dry season and estivation. A seasonal pattern has also been reported in other freshwater crab species. for instance, a year round study of male primary freshwater crab gonad *Candidiopotamon*

rathbunae (Potamidae) shows the peak of GSI during high precipitation (Liu and Li, 2000).

Mandibular organ (MO) is one of reproductive endocrine organs that produce hormone responding to environment. MO mainly secrete the methyl farnesoate under the control by X-organ-sinus-gland (see pathway in Chapter II). The methyl farnesoate is known to stimulate testicular growth (Nagaraju et al., 2004; Reddy et al., 2004). The activity of MO has been investigated based on the histology of MO cells. Dense and less dense cells in MO cells has been identified in *Fenneropenaeus indicus* (Penaeid shrimps) (Vijayan and Diwan, 1994). Based on cell and nuclei size, two different cell types has been recognized in *Travancoriana schirnerae* (Gecarcinucidae, primary freshwater crab) (Gopal and Devi, 2018). In the present study, unfortunately, the structure of MO in *E. nani* cannot be identified. With the obscure location in cephalothorax, other accessory organ nearby mandibular muscle were found instead (Appendix A). Since the tubular structure of this accessory organ did not to fit with the general structure of endocrine organ, it is certain that the microstructure found in this organ cannot be interpreted as MO in *E. nani*. Therefore, other accessory organs nearby mandible area of *E. nani* should be further investigated.

Active testicular structures were found in wet season, while the resting testicular structures were recognized in dry season. The low activity of testis remained in the resting testicular stage. This aspect link to an ability of spermatozoa storage in MVD so that the spermatozoa are presented year-round. It would be a beneficial strategy for a male to set gonadal maturation, in order to copulate with females that increases their chances of success during breeding in wet season. Furthermore, lack of

spermatophore formation and direct habitation in seminal fluid of spermatozoa could be related with the reproductive strategy of the crabs producing less offspring but with longer parental care and a nearly direct development (*r*-strategy).

CHAPTER V

Morphological and Physiological Evidence of Two Annual Reproductive Peaks in the Female Rice Field Crab *Esanthelphusa nani* (Naiyanetr, 1984)

INTRODUCTION

True crabs (infraorder Brachyura) are a very diverse decapod crustacean group with up to 7,000 species. Marine, secondary freshwater, and primary freshwater crabs are generally classified based on habitats of larva and adults: marine crabs spend all their life in marine environment, primary freshwater crabs are exclusively freshwater or terrestrial and secondary freshwater crabs start their development in the sea but spend the rest of their life in continental environment (Ng et al., 2008; De Grave et al., 2009). The marine group is the most diversified in terms of species number (Ng et al., 2008) and evolution to freshwater may have occurred several times in the group as revealed by recent molecular based phylogenies (Cumberlidge et al., 2009; Tsang et al., 2014).

This iterative evolution makes them an interesting group to appreciate how convergence related to habitat shift. The evolution of reproductive strategies in this respect are interesting, because primary freshwater crabs tend to have a more direct postnatal development than other marine crabs. Sea-to-land transition, primary freshwater crabs have to adjust their physiology for presence on land environments and adapt reproductive strategy to produce offspring in an unpredictably limited water environment (Augusto et al., 2007). Reduce egg numbers, provide maternal care and switch from *r*-strategy to *k*-strategy are part of the primary freshwater crab's

reproductive adaptation against land environments (Vogt, 2013). In this respect, studying early phases of development, sexual organs and germinal cycle can be particularly interesting (Smija and Devi, 2015; Sharifian et al., 2015., Sun et al., 2018), however, there are still few data concerning the oogenesis and ovarian cycle for primary freshwater crabs. Moreover, there is a few known on comparative data of ovarian development of marine and freshwater crabs that might reflex the reproductive adaptation to environments.

Basically, the reproductive tract in female crab is very similar among marine and freshwater crabs (de Souza and Silva, 2009; Keunecke et al., 2009; Islam et al., 2010; Smija and Devi, 2015). It displays an H or X shape in cephalothorax. A pair of ovaries connected by commissure bridge is present above hepatopancreas. A pair of short oviducts connects the posterior end of ovaries and a pair of spermatheca. A pair of vaginas connect the spermatheca and the paired genital opening at 6^{th} sternite plate (Guinot et al., 2013). Many studies have shown similar patterns of ovarian development among crabs based on macroscopic coloration and size of ovary (Lima et al., 2006; Rotllant et al., 2007; da Silva et al., 2012; Shinozaki-Mendes et al., 2012b). Together with macroscopic examination, histological approaches have also been widely used for verifying gonadal growth and maturation in female crab (Leme, 2006; Rotllant et al., 2007; Keunecke et al., 2009; Islam et al., 2010; Sarker and Kumar, 2013; Sharifian et al., 2015; Sun et al., 2018). During reproductive cycle, the gonads of female crab display a series of morphological changes. Histological differences can be noticed concerning the number of oocytes and how packed they are during specific cellular differentiation phases. (Sharifian et al., 2015; Smija and Devi,

2015). The series of oocyte development in crustacean requires hormonal controls that relate to the pathway of yolk protein deposit (Nagaraju, 2011).

In egg-laying animals, vitellogenin (Vtg), a yolk-protein precursor, is produced in specific tissues and subsequently accumulated in the ovary before modified into vitellin (Meusy and Payen, 1988b). Then, vitellin is accumulated to oocytes which results in an increasing in the diameter of the oocytes (Tsukimura, 2001). Vtg is classified as a large lipoprotein molecule that has a vary size depending on crustacean species (Khalaila et al., 2004). Various Vtg synthesis sites have been documented in crustaceans in different species. Crustacean Vtg was expressed mostly in ovary and hepatopancreas (Tsang et al., 2003; Mak et al., 2005; Yang et al., 2005). Several studies have revealed that detectable hemolymph vitellogenin levels change in relation to ovarian development in crustacean species (Wilder et al., 2002). It is thus interesting to test whether crab has a vitellogenin cycle in relating to ovarian development.

Esanthelphusa nani, the rice field crabs described based on specimens caught at Nan Province in Thailand, is both essential economic benefits and problem in local area. Nevertheless, there is no previous data of ovarian development in morphological and histological description of the rice field in Gecarcinucidae family. This study here focuses on the description of the macrostructure and histology of female sex organs of *E. nani*. Since high relationship between ovary growth and Vtg levels, the study provides Vtg level monitoring to understand the descriptions of the anatomy of the reproductive organs of the female and its change during the year.

MATERIALS AND METHODS

Crab sampling

The study site located at the rice field at Lai Nan sub-district, Wiang Sa district, Nan province (see Chapter III). A field survey was carried out from dusk to late night. All samplings were done from November 2015 to November 2016 on a monthly basis. Every month, five adult female crabs with a carapace width greater than 30 mm were caught by hand. After that, the crabs were transported to a local laboratory and acclimatized till the next day. Then, the crabs were euthanized in ice slurry and later weighed and measured for carapace width. The crabs were afterward dissected, and hemolymph was collected directly by cardiac puncture. Then, 250mM of critic acid was added to the same quantity of hemolymph to prevent it from clotting. The hemolymph was kept at -20 °C for alkali–labile phosphate analysis.

Macroscopic examination

Once the carapace was dissected off, the heart and digestive tract were carefully removed. Then the majority of the reproductive tract was located and observable over the hepatopancreas and the ovary could be photographed in its cephalothorax cavity. All crabs were photographed using the same conditions with a Nikon d750 camera and an AF-d 60 mm lens to deliver a fine detail picture with less distortion. The color of white balance was calibrated and set to all pictures. Ovarian stages were described based on the volume of the ovary relative to the cephalothorax cavity and on changing of coloration. After the photograph, the ovary was gently removed out of cephalothorax cavity and subject to gravimetric study. Gonadosomatic index (GSI) was calculated using the following formula:

GSI = [the weight of ovary /total weight of crab] × 100

Finally, the whole ovary was fixed in Davidson's solution overnight. Seventy percent ethanol was used as a preservative solution prior to histological procedures for microscopic examination.

Microscopic examination

As ovarian tissues showed a similarity along the ovary (anterior and posterior horn of left and right sides), only left anterior horns for 3 different crabs sampled in each month of the year were observed in detail under the microscope, with sections of 7 μM in order to assess histological changes during the ovarian cycle. The ovary section was stained by hematoxylin and eosin (Appendix B). All tissues were explored under light microscope and recorded by Canon 7DII fitting on the scope. Pictures were merged in order to depict the whole section of the anterior horn of ovary. Different stages of gametogenesis were estimated from the shape of tissues and oocytes. For each stage that would be defined, 3 different individuals were observed in order to appreciate interindividual variation.

Change in vitellogenin concentration during the ovarian cycle

The approach of Gagné and André (2011) to estimate change in vitellogenin (Vtg) proportion in hemolymph was followed in this study. For that, the free phosphates of the lipophosphoprotein were first released by NaOH hydrolysis in the organic phase. Then, the detection of free phosphates was assessed by using Phosphate Assay Kit (Sigma, Catalog Number MAK308). Finally, the concentration of free phosphates was normalized by comparing them to the total proteins in the sample.

Since several phospholipoproteins can be found in hemolymph, detectable level of free phosphate released from the phospholiproprotein thus represents level of vitellogenin together with other phopholipoproteins in the hemolymph. Therefore, in this study, hemolymph of male crab which is normally devoid of vitellogenin were used to check for the background level of the constitutive phospholipoproteins in hemolymph. The representative samples of male hemolymph (Nov 2015, Feb 2015, May 2015, Aug 2015, and Nov 2016; N = 3 crabs/month) were extracted and determined for ALP concentration using the same method as the female hemolymph.

Hemolymph total protein determination

In order to estimate total concentration of protein in hemolymph, Bradford's assay (Bradford, 1976) was employed. Firstly, the stock of 25 µg/mL bovine serum albumin (BSA) was prepared from 2 mg/mL of commercial BSA (Thermo®). The BSA standard and hemolymph were diluted by phosphate-buffered saline. Serial dilution of the stock BSA was prepared to get a concentration from 0.78125, 1.5625 ,3.125, 6.25, 12.5, and 25 µg/mL. Hemolymph was diluted at 1:200 and 1:400. A hundred µL of standards, blanks, and samples were added in duplicate into each well. A 100 µL aliquot of Bradford solution (Sigma®) was added in duplicate into the individual wells. The assay plate was tapped to mixed well before measuring for absorbance at 595 nm with a microplate reader (Multiskan EX).

Ether extraction

In this procedure, ether extraction was used for protein extraction. Crab hemolymph was diluted at a 1:200 ratio with saline solution to adjust a suitable protein concentration for extraction. The 250 µL of diluted crab hemolymph was then extracted with 250 µL of *t*-butyl methyl ether. Components were mixed vigorously

for 15 min on a shaker to reach perfect emulsion. The emulsion was centrifuged at 10,000 ×g for 5 min to isolate the organic phase from the aqueous phase. Two hundred μL of the organic phase was collected for the liberation of phosphate.

Liberation of phosphate

The organic phase was mixed vigorously with 200 μL of 2 M NaOH for 5 min. Then emulsion was incubated in a heat block at 37 °C for 10 min. The emulsion was subjected to repeat the two previous procedures for 3 times. Then, an aliquot of 150 μL was transferred for phosphate determination in the aqueous phase.

Determination of free phosphate

In this respect, a premix at 40 μM for standard dilution was prepared freshly from 1 M phosphate standards stock. The 40 μM premix was diluted serially into 32, 24, 16, 12, 8, and 4 μM for making a standard curve of ALP assay. A 50 μL aliquot of the serial standards or liberated sample was added in duplicate into wells of the microplate. Ultrapure water was added instead of the blank of the assay. The Malachite Green Reagent (SIGMA®) was added into each well. The plate was then gently tapped, and incubated for 30 minutes at room temperature for color development. Finally, the absorbance of the assay plate was measured at 620 nm on a microplate reader (Multiskan EX).

Data analysis

Oocyte phases and ovarian stages were first identified from the observation of the whole sample. Change in GSI and ALP qualitative data were established month by month. Monthly GSI and ALP data were checked for normal distributions before multiple comparisons were performed. One way ANOVA followed by the Tukey

post-hoc test, was used to compare GSI. Kruskal-Wallis followed by the Dunn post-hoc, on the other hand, was used to estimate ALP change as these data were not normally distributed. The Spearman rank correlation test was done to estimate any correlation between GSI and ALP.

RESULTS

General descriptive appearance of E. nani ovary (Fig. 5.1)

The female reproductive tract of *E. nani* is an 'H' in shape. The tract consists of a pair of ovaries and spermathecae. The left and right sides of the ovary are connected by the commissure bridge at the center. Anterior ovaries usually locate below stomach. Eliminating the stomach reveals the anterior ovaries laying on the hepatopancreas. Posterior ovaries dive deeply under the heart region. The end of posterior ovaries is linked with spermathecae by oviducts at the level of the third abdominal segment. Dorsal spermathecae are atriums, which are used for sperm storage. On the ventral side of spermathecae, there is a pair of short vaginas which end to the genital opening located on the 6th sternite plate of cephalothorax. The color and size of ovaries depend on stage of development that exhibits in white, light yellow, golden and orange.

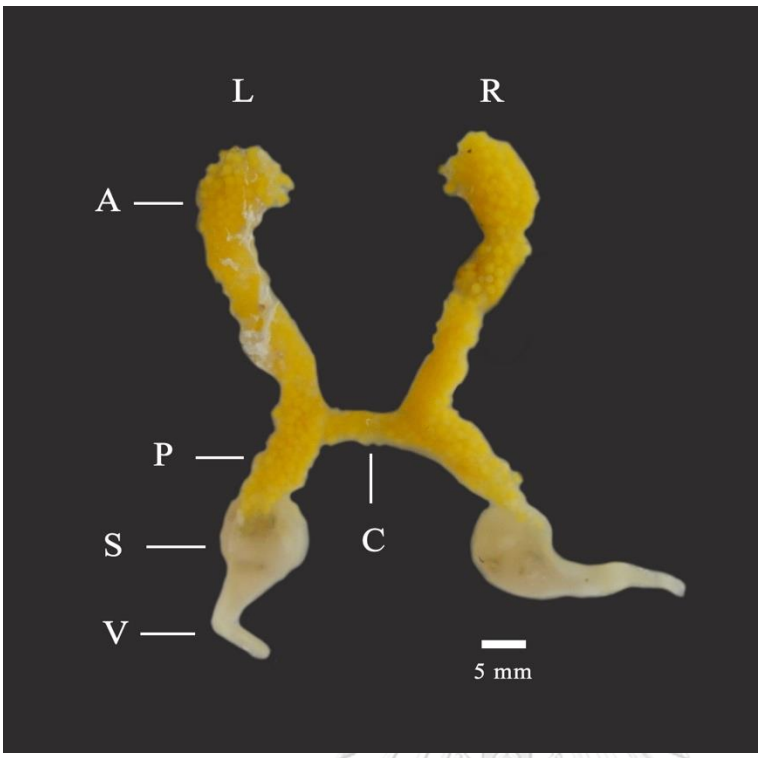


Figure 5.1 Overview of internal reproductive tract of *Esanthelphusa nani* shows in an 'H' shape which consists of ovary and spermatheca. A: anterior ovary; C: commissure bridge; R: Right side; L: Left side; P: posterior ovary; S: spermatheca; V: vagina.

Oogonia and phases of oocyte

Oogonia (Fig. 5.2A)

Clusters of oogonia (Oo) are present at the germinal zone (GZ) of the ovary. Oogonia are round cells. The large and round nucleus contains heterochromatin that stained deep basophilic color. The nucleolus is not observed at this stage. There is little cytoplasm (hazy blue in our colorations). Oogonia have a diameter of 23.51 ± 3.16 μm (N = 3 crab, N = 20 cells/crab).

Growing oocyte (Phase 1 oocyte; Fig. 5.2B)

Phase 1 oocytes (Oc1) are usually present next to the germinal zone. Oc1 are cells of more irregular outlines than the oogonia. The oval nucleus increases in size and the nucleoplasm has a small granule. One or two large nucleoli are present at this

stage. The basophilic cytoplasm grows, and cytosol increase relative to the nuclear part. Flatten nucleus follicular cells (fc) appear to surround the Oocyte at this phase. Oc1 is 327.03 ± 74.23 μm in diameter (*N* = 3 crabs, *N* = 20 cells/crab).

Pre–vitellogenic oocyte (Phase 2 oocyte; Fig. 5.2C)

Phase 2 oocytes (Oc2) are also located nearby the germinal zone. Oc2 are round or polygonal cells. The nucleus shows several small nucleolar granules around one or two big nucleoli. With our colorations, the cytoplasm of Oc2 exhibits a reddish color due to eosinophilia. The cytoplasm size and volume continue to increase. As a result, cytoplasmic–nucleoplasm ratio is greater than in the two previous stages. Follicular cells (fc) at this stage completely surround the Oc2. Oc2 is 440.44 ± 42.63 μm in diameter (*N* = 3 crabs, *N* = 20 cells/crab).

Early–vitellogenic oocyte (Phase 3 oocyte; Fig. 5.2D)

Phase 3 oocytes (Oc3) are found near the Oc1 and Oc2 zone. Nucleus shows one or two big nucleoli. The nucleolar granules in nucleoplasm exhibit an eosinophilic color. The cytoplasm begins to be differentiated into 2 zones. A first cytoplasmic zone (CZ) is characterized by the homogeneity eosinophilic cytoplasm. While a vitellolysis zone (VZ) consists of large yolk granules and lipid droplets which are the essential nutrient of yolk. Surrounding follicular cells (fc) are presented. Oc3 is 808.38 ± 52.55 μm in diameter (*N* = 3 crabs, *N* = 20 cells/crab).

Late–vitellogenic oocyte (Phase 4 oocyte; Fig. 5.2E)

Phase 4 oocyte (Oc4) are found in the same location as Oc3. One or two big nucleoli are present in the eosinophilic nucleoplasm. The cytoplasm is well differentiated into 2 zones. The major character of Oc4 is that the portion of vitellolysis zone (VZ) is greater than the portion of cytoplasmic zone (CZ). In the

vitellolysis zone, two vesicle types can be classified yolk granules which show reddish stain and lipid droplets which show empty round spot. Surrounding follicular cells (fc) present. Oc4 is 1113.39 ± 135.08μm in diameter (*N* = 3 crabs, *N* = 20 cells/crab).

Mature oocyte (Phase 5 oocyte; Fig. 5.2F)

Phase 5 oocytes (Oc5) are found in the same zone as Oc4 and Oc3. The condensed nucleus is compressed by yolk platelets. The nucleolus is difficult to observe. The nucleoplasm is dark. Oc5 is full of yolk platelets. The vitellolysis zone occupies all the cytoplasm. The follicular cells (fc) are in a flat shape. Oc5 is 1487.33 ± 89.86 μm in diameter (*N* = 3 crabs, *N* = 20 cells/crab).

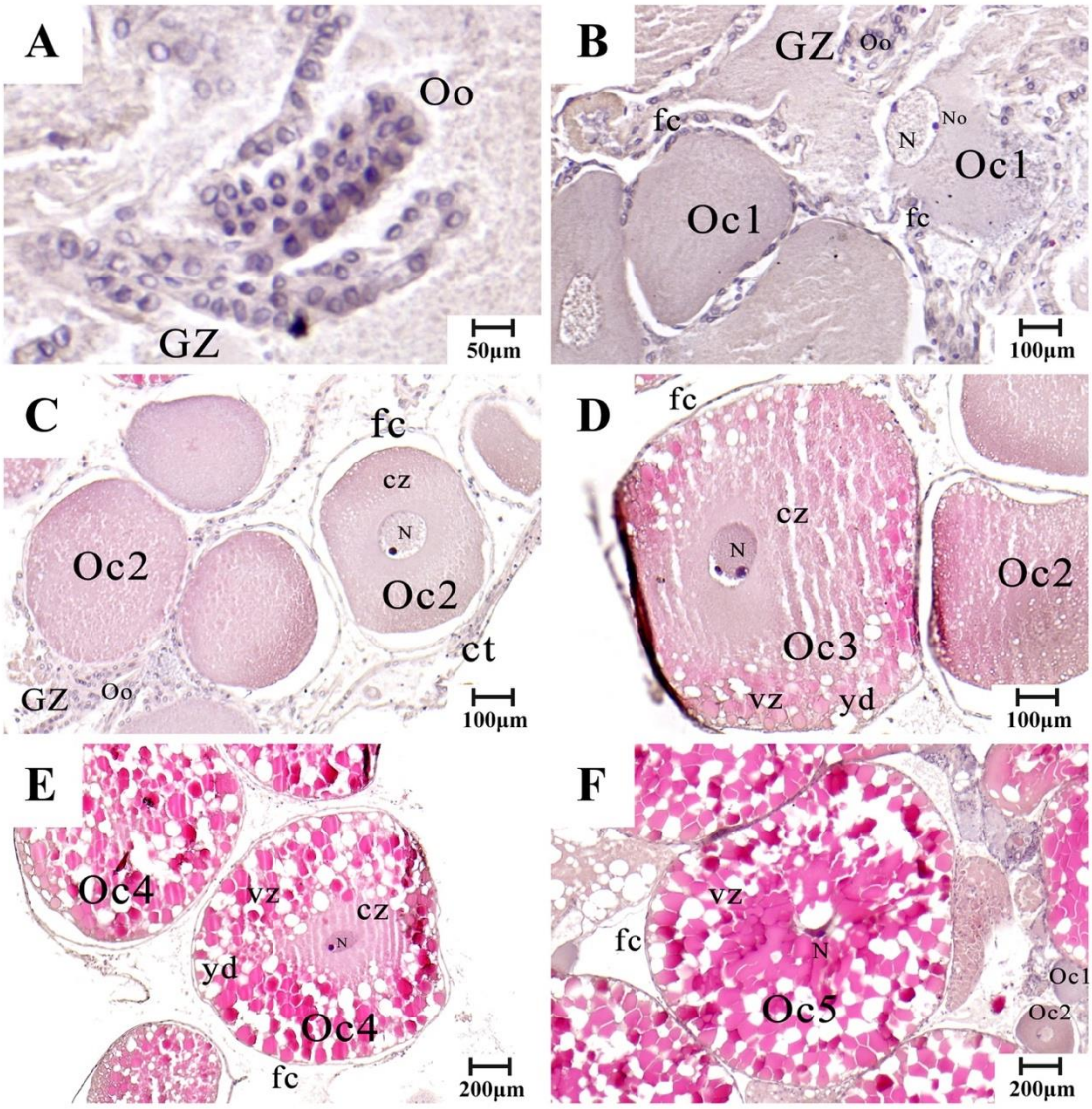


Figure 5.2 Micrographs of differentiating oocytes in different phases. A) oogonia; B) phase one oocytes with basophilic and irregular cytoplasm; C) second phase oocytes with eosinophilic cytoplasm; D) third phase oocytes with beginning of vitellogenesis; E) fourth phase oocyte with vitellolysis zone occupied more than 50% of the cell; F) fifth phase oocytes with full of yolk droplets. ct: fibro connective tissue; cz: cytoplasmic zone; GZ: germinal zone; fc: follicular cell.; N: No: nucleolus; nucleus; vz: vitellolysis zone; yd: yolk droplets.

The ovarian development stages

Stage I resting stage

Resting stage ovary was found from September to November. The gametogenesis can be found at a resting stage. The hepatopancreas present yellow color and its largest portion is located in the cephalothorax. The ovaries are smallest at that time. Their sizes are about 3% (N = 10) of cephalothorax cavity. Their color is creamy white (Fig. 5.3A, top). The germinal zone presented throughout the ovarian tissue consists of abundant oogonia (Fig. 5.3A, below). Phase I and phase II oocytes are dominating the germinal tissues. Finally, the ovarian tissue is full of unsuccessful and distorted oocytes. At this period of the year, oocyte development does not progress after phase II. Collapsed oocytes are re-absorbed by the ovary (Fig. 5.4).

Stage II Vitellogenic stage

From December to January, ovarian sizes increase. The sizes reach about 10% (N = 10) of cephalothorax cavity. The external texture of ovary is granular. Their color is light yellowish (Fig. 5.3B, top). The germinal zone is in the middle of the ovary with mostly Oc1, Oc2 (Fig. 5.3B, below). A few Oc3, Oc4 distribute distinctly from the germinal zone. The dominant oocyte type is phase 2 oocytes (Fig. 5.4). This stage is called vitellogenic stage since oocytes start to acquire more and more vitellin.

Stage III Pre–mature stage

A pre–mature stage was found during two periods: from February to April and again from June to August. The ovary has a much larger size that can occupy 20% of cephalothorax cavity (N = 10). The tiny oocytes was observable on the surface of the ovary with naked–eyes. Ovary color exhibits deep yellowish color (Fig. 5.3C, top). The germinal zone with Oc1, Oc2 presents in the center of ovary (Fig. 5.3C, below).

The late vitellogenesis oocytes at phase 4 (Oc4) displays predominantly around the ovary. Several Oc5 are also present at this stage (Fig. 5.4).

Stage IV Mature stage

Mature stages can be found during two periods of the year: from February to April and from June to August. This stage can be defined by the massive ovarian size occupying at 20% (N = 10) of cephalothorax cavity. The tiny oocytes turn at the surface of the ovary are orange in color and observable by eyes; the ovary is orange in color as well (Fig. 5.3D, top). The germinal zone is present in at a tiny space in the center of the ovary tissue (Fig. 5.3D, below). Mainly, mature oocytes (Oc5) are existing throughout the ovarian tissue (Fig. 5.4).

Stage V Spawning and regenerating stage

Crabs in spawning and regenerating stage can be observed between May to August. Ovarian sizes decrease to about 10% of cephalothorax cavity (N = 10). The different colors and patterns of macroscopic pictures distinguish these two stages from the others. At Spawning and regenerating stage, the oocyte on the surface of the ovary varies in color but can be seen by naked–eyes (Fig. 5.3E, top). The ovarian appearance exhibits a general white color with yellow dots. Histological section shows that the ovarian tissue consists of numerous Oo1, Oo2 and Oo3 (Fig. 5.3E, below). The germinal zone is very active at this stage.

Figure 5.3 Macroscopic view and microscopic view (explanation shows in the next pages)

Figure 5.3. Macroscopic view (top row) and the high magnification view (middle row) of different stages of ovarian development. Ovaries were classified into stage I: resting stage, stage II: vitellogenic stage, stage III: pre–mature stages, stage IV: mature stage and V: Spanwing and regenerating stage. The overview microscopic view (bottom row) shows different oocyte phases distributing in those ovarian stages. The red lines in the middle pictures present the plane of ovarian section. GZ: germinal zone, Oc1–5: oocyte phase I–V; OD: distorted oocytes.

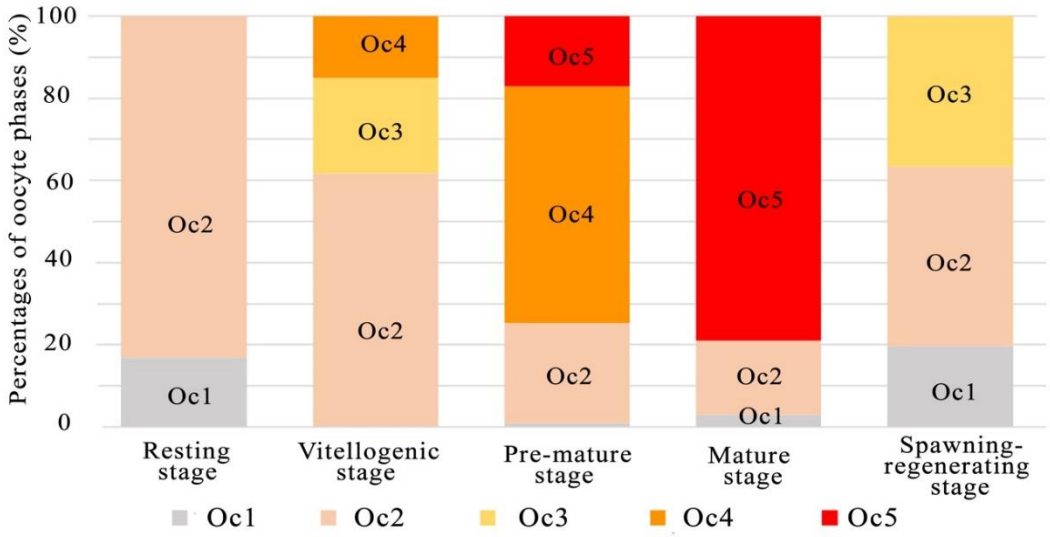


Figure 5.4 Proportion (percent) of five oocyte phases in each ovarian stage (N = 3 /stage). Oc1: first phase oocyte; Oc2: first phase oocyte; Oc3: first phase oocyte; Oc4: first phase oocyte; Oc5: phase oocyte.

Variation in on gonadosomatic index during the year (GSI)

The climate in the Northern part of Thailand can be divided as dry and wet seasons during 2015 to 2016 (see Chapter III). November 2015 to April 2016 and October 2016 to November 2016 corresponded to dry months, while May to September 2016 corresponded to the wet months of the study period. The present study was able to show significant monthly variation for GSI of the female crabs (ANOVA, $p < 0.05$) (Fig 5.5A). The GSI gradually increased during the dry season from October to reach a peak in February (estivation period since there is no more water in rice fields at that time). Then, GSI gradually decreased until May at the end of dry season before re-increasing again in June and August during the wet season (Tukey post hoc test, $p < 0.05$) and to decrease again from September. Therefore, two

peaks of GSI could be identified in February and in August, suggesting that crabs enter two reproductively active times in each year.

Biological free phosphate determination by alkali–labile Phosphates (ALP)

The free phosphate concentration in hemolymph of the male crab ranged between 0.9 to 4.9 μmole of phosphate/mg total protein. There was no significant seasonal difference of male ALP (The Kruskal-Wallis, $p > 0.05$) (Fig 5.5B). Thus, the male ALP was grouped and calculated for the mean value of 2.7 μmole of phosphate/mg total protein. This background ALP value was then used as the refence line or baseline of the constitutive ALP level in crab hemolymph extraction (Figure 5.5C).

The free phosphate concentration in hemolymph of the female crab ranged between 0.9 to 19.3 μmole of phosphate/mg of protein. The data was failed on homogeneity test. The Kruskal-Wallis did not show any significant difference of ALP ($p>0.05$) because data exhibited a very high intra-month variation (Fig. 5.5C). The Spearman's rank correlation showed a weakly positive relationship between GSI and ALP ($r_s = 0.18$, $p = 0.17$). And the trend of ALP was grossly corresponding to GSI (Fig. 5.5D). In particular, the ALP curve showed two peaks that fairly match the one of GSI in February and June. In addition, it should note that the two pecks of ALP are higher than the ALP baseline.

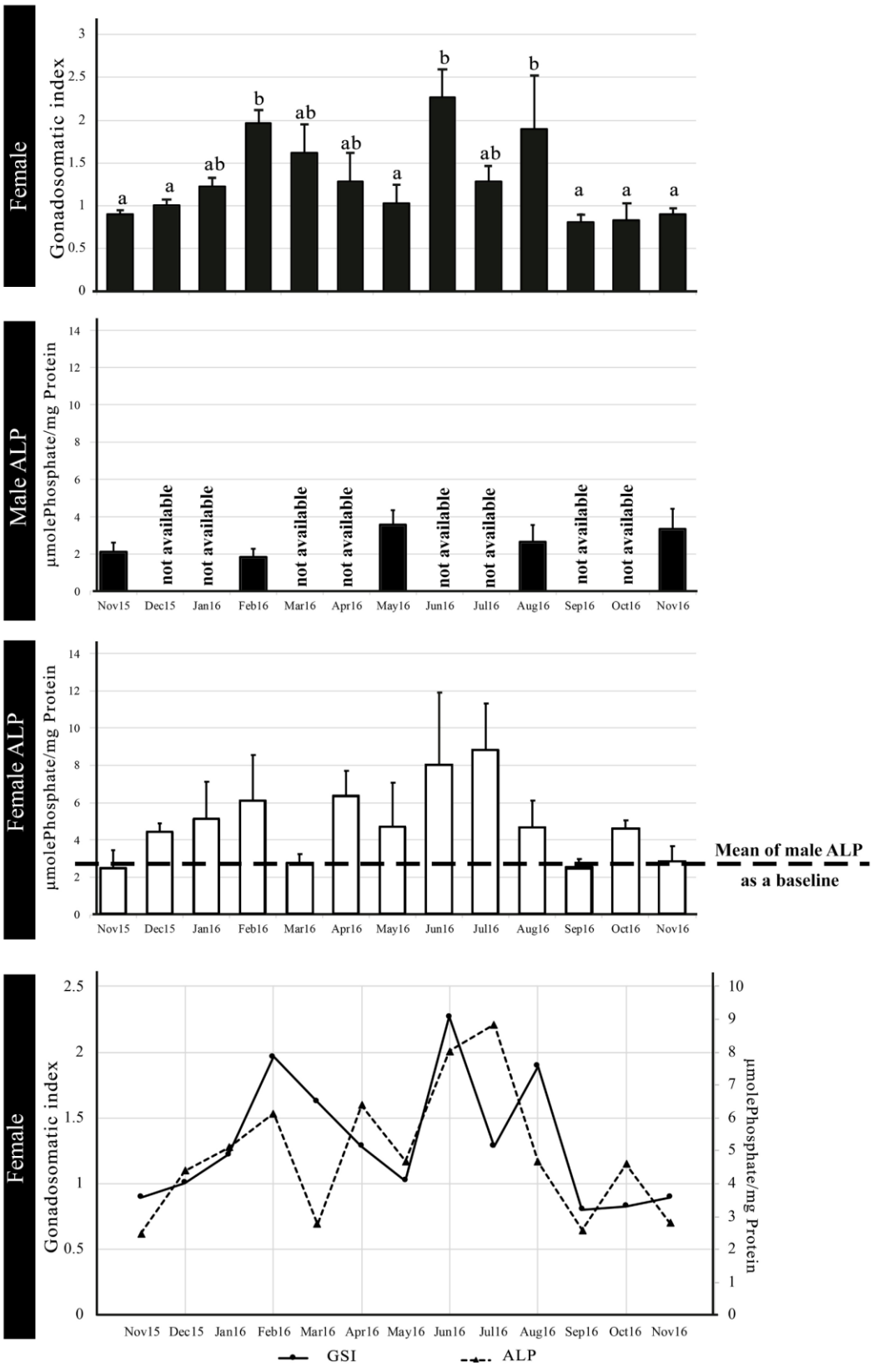

Female
Gonadosomatic index
a
a
ab
b
ab
ab
a
b
ab
b
a
a
a
Male ALP
µmolePhosphate/mg Protein
not available
Female ALP
µmolePhosphate/mg Protein
Mean of male ALP
as a baseline
Nov15
Dec15
Jan16
Feb16
Mar16
Apr16
May16
Jun16
Jul16
Aug16
Sep16
Oct16
Nov16
Female
Gonadosomatic index
µmolePhosphate/mg Protein
GSI
ALP

Figure 5.5 A) Seasonal variation of gonadosomatic index of female rice field crab, *E. nani*, based on monthly sample. The significant differences of GSI are shown in different alphabets (ANOVA, Tukey HSD post-hoc, p=0.05); B) Phosphate liberation levels of lipoprotein extraction or ALP assay in the male crab hemolymph; C) Phosphate liberation levels of lipoprotein extraction or ALP assay in the female crab hemolymph corresponds to GSI sampling; D) Plots between GSI and the ALP shows trend of an association between ovarian development and amount of phospholipoprotein in hemolymph.

DISCUSSION & CONCLUSION

A common and quick evaluation of ovarian development stages in brachyuran is macroscopic examination of ovary (Mantelatto and Fransozo, 1999). The technique shows strong association of oocyte development (Sun et al., 2018). Color changes of yellowish ovary display directly an outcome of oocyte vitellogenesis that associates to carotenoids and retinoids (Liñán-Cabello et al., 2002). General descriptive ovarian stages of *Sodhiana iranica* shows four fundamental development stages: I. resting, II. proliferative (vitellogenic) III. pre–mature and stage IV. mature stages (Sharifian et al., 2015). The four stage names are sometimes used as a synonym in *Paratelphusa lamellifrons*: I. immature II. early mature III. late mature and stage IV. ripe stages (Sarker and Kumar, 2013). Those studies are however similar in character and coloration pattern. Five developmental stages of ovary with two vitellogenic stages ovary has reported in *Uca rapax* (Castiglioni et al., 2007). Moreover, six ovarian stages of ovarian development *Sinopotamon henanense* have been intensively divided. The gradual increase in size and changing in color correspond to oocyte phase has been observed (Sun et al., 2018). In this study, changes in ovarian size and color were associated with a number of oocyte development. This study tries to simplify the macroscopic examination as a common and quick evaluation method for investing ovarian stages. There were presented in four common stages, while the fifth stage, spawning and regenerate stage, was unique performance in *E. nani*. It could increase a change for producing the offspring more than one time a year.

Histological analysis is a basic technique to identify the development of oogonia and oocyte stages in marine and freshwater crabs (Islam et al., 2010; Sharifian et al., 2015). Microscopic difference of characteristic oocytes is a standard

investigation of oocyte maturation (da Silva et al., 2012). The pack of oogonia of *U. rapax* usually presents in the germinal zone located in the center region of ovary (Castiglioni et al., 2007). The freshwater crab *S. henanense* shows several cords of germinal zone reported along the middle of ovary (Sun et al., 2018). The changing of cytoplasm from basophilic to acidophilic color is a sign for starting phase of vitellogenic oocyte. Those criterions are common in recognizing early stage of oocyte in brachyuran (Minagawa et al., 1993). The discrepant oocyte phase is however the phase after vitellogenic oocyte. The basic classification of germ cells *Goniopsis cruentata* and *Cardisoma guanhumi* are splitted into four phases: oogonium, previtellogenic oocyte, vitellogenic oocyte, and mature oocyte (de Souza and Silva, 2009; Shinozaki-Mendes et al., 2012a). While six oocyte phases with one oogonia phase is reported in *S. iranica* and *S. henanense* (Sharifian et al., 2015; Sun et al., 2018). Interestingly, using the combination of staining was able to investigate oocytes up to ten phases including oogonia (Smija and Devi, 2015). In the current study based on hematoxylin and eosin staining, the five phases of oocytes with one oogonia of *E. nani* were classified based on criterion of cytoplasmic changes.

The ovarian stage of crabs is shown directly by GSI because the growing oocyte generates a gain in ovary size and volume (Minagawa et al., 1993). Also, vitellogenin is highly associated with vitellin synthesis, which results in oocyte growth (Polzonetti-Magni et al., 2004). The hemolymph Vtg levels gradually increase during vitellogenesis of the ovarian stage, and then the levels decrease in the mature stage of ovary (Zmora et al., 2007). By that meaning, the GSI and Vtg levels are in the same at vitellogenic stage, but they are distinct at the mature stage of the ovary. In the rice field crabs, *E. nani*, it shows the increasing tendency of ALP levels (Fig.

5.5D) that corresponding to vitellogenic stage. The decreasing tendency does not however show clearly district between the GSI and Vtg levels. Interestingly, the period between June to August presented different rising level of ALP from other months. It could infer that the vitellin synthetization rate which proceeds of oocyte growth during the time, might be the most active in a year. Indeed, the hormonal mechanism of regulation of vitellogenesis is still needed to consider.

The cycle of seasonal breeding in crabs varies and can be explained by divergent taxonomic relations and ecological niches. The marine crab, *Hapalocarcinus marsupialis*, shows potentially continuous breeder around the year (El-Damhougy et al., 2018). The freshwater crab, *S. henanense*, shows a seasonal breeder which have one spawning season during May and June (Sun et al., 2018). The coast crab, *Sesarma rectum*, shows two periods of spawning egg in a year (Leme, 2006). The study of GSI indicated that *E. nani* is a possible seasonal breeder that has more than one spawning season in a year. One occurred in February, dry season. Then, at least another one, the spawning occurred during wet season, June to August. Moreover, according to the ALP result, the rate of reproductive production in the wet season was potentially higher than those in dry season. The study of *E nani* could be emphasized that the variations in the breeding season of brachyuran could due to the genetic variety and the residential environments.

CHAPTER VI

Embryonic Development of the Rice Field Crab

Esanthelphusa nani (Naiyanetr, 1984)

INTRODUCTION

One fundamental aspect of ecological–evolutionary–developmental biology (eco-evo-devo) is to understand how ecology may interfere with development and evolution and how phylogenetic history and development may constrain ontogenies (Gould, 1977; Gilbert and Epel, 2015). The evolutionary transition between environments is particularly interesting in this respect. The aquatic–terrestrial transition in vertebrates is a well–known example. The amniotic egg is a developmental innovation that allowed that transition and that is accompanied by remarkable changes in the early ontogeny (Sumida and Martin, 1997). The amniotic egg has also been acquired independently in arthropods and is related to the colonization of the terrestrial environment in this group about 444–457 million years ago (Dunn, 2013). While the development of land and aquatic arthropods is relatively well documented, the information on the different steps of evolutionary change during that transition is poorly discussed. This is mostly because low taxonomic ranks of arthropods are inflowed to a single environment and a given development. Crustaceans and crabs in particular, however, offer the possibility to understand that transition from a comparative point of view because they evolved iteratively to freshwater and the continental environment from marine forms (Jagt et al., 2015). On the other hand, crab development is well documented for marine groups, but the ontogeny of terrestrial and freshwater forms has been less studied.

The infraorder Brachyura (True crabs) is an extremely diversified group with 7,000 species (Cumberlidge et al., 2009) they can be classified into three major ecological groups: marine, secondary freshwater and primary freshwater crabs. Marine crabs spend all their life and development in the sea, secondary freshwater crabs start their development in the sea and migrate later in their development to land, and primary freshwater crabs (or true freshwater crabs) do their entire development on land. In this group, the development is largely modified, with direct development, production of fewer eggs and apparition of parental care. As a consequence, some crabs of this group can become fully terrestrial (Vogt, 2013). The ontogenetic sequence has been documented in most ecologic and taxonomic groups. A simplified phylogeny derived from the work of Tsang et al. (2014) is provided in Fig. 6.1. Primitive marine crabs (Raninidae) present more than 6 zoea stages and one megalopa stage (Minagawa and Murano, 1993). Eu–marine crabs (e.g., Portunidae, Grapsidae, Epialtidae, etc.) have 4–6 zoea stages and one megalopa stage (Dornelas et al., 2004; Islam et al., 2005; Oh and Sook Ko, 2010). At least five families evolved as secondary freshwater crabs (Gecarcinidae, Varunidae, Sesarmidae, Pinnatheridae and some Chasmocarcinidae) and their development is very similar to eu–marine crab with zoea and megalopa stages (Cuesta et al., 2007; Kornienko et al., 2008; Cuesta et al., 2011). However, an abbreviation of larval development (with a reduction of stage number) has been documented in some of these secondary freshwater crabs (Bolaños et al., 2005). Freshwater crabs have been classified into 5 families (Potamonautidae, Gecarcinucidae, Potamidae, Pseudothelphusidae, and Trichodactylidae) but their development has rarely been studied. This is mostly because the development is direct and hatchling already displays adult–like morphology (Ng et al., 2008). Pace et al.

(1976) was probably the first to provide an idea of the embryonic development of these crabs in his study on the potamid crab *Potamon edulis* using histological sections. Later, Wu et al. (2010) documented the ontogenetic sequence within the egg in the potamid crab *Sinopotamon yangtsekiense* using stereomicroscopy but could not identify several features of the development such as earliest developmental stages or appendage fine anatomy. This kind of studies are nevertheless crucial for documenting and understanding the links between development, ecology and crab morphological evolution.

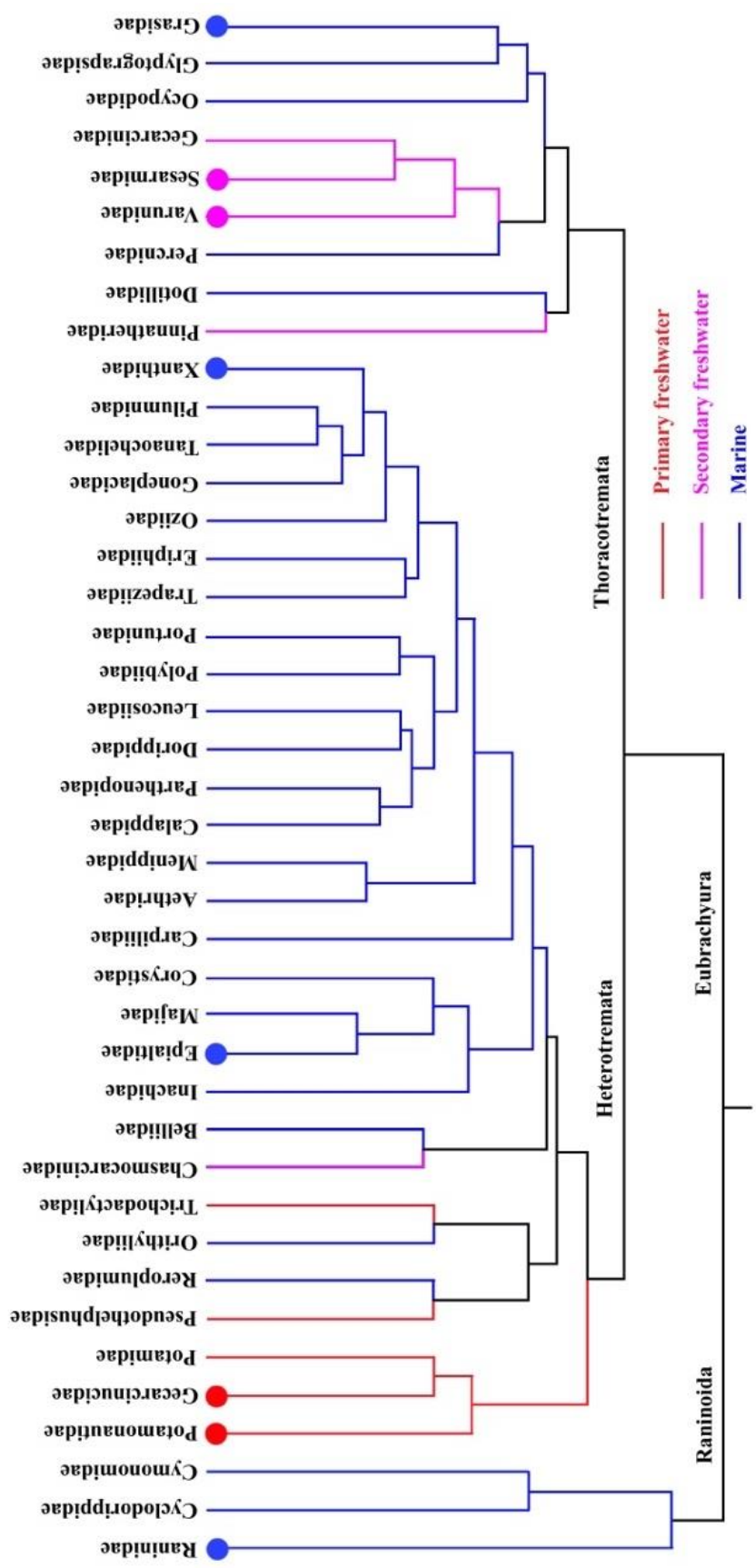


Figure 6.1 Phylogenetic relationships between members of the brachyura family; modified tree based from analyses of genetic data sets by Tsang et al. (2014). The different color presents the three major ecological lifestyles: primary freshwater, secondary freshwater and marine crabs. The circle marks on the tree are those families for which embryonic and larval development data are present in **Table 6.1**.

As said previously, primary freshwater crabs are diverse, and the study of their development could shed important light on evolutionary transition in this group. In this context, the development of a true freshwater crab, the rice field crab *Esanthelphusa nani* of the Gecarcinucidae family was analyzed. This crab was firstly described based on specimens caught at Nan Province in Thailand (Naiyanetr, 1984), and understanding its development can also have an important impact for controlling its population in the wild because it is considered as a pest destroying rice production or as a local food resource (Rajasekaran and Whiteford, 1993). Also and for the first time, the development of this freshwater crab were carried out with both stereo and confocal imaging which provides a more detailed analysis of its direct development.

MATERIALS AND METHODS

Egg sampling

The material was obtained in the rice fields of the Lai Nan sub-district, Wiang Sa district, Nan province, where the endemic species of the rice field crab, *E. nani* is present (see Chapter III). Most of the ovigerous crabs were caught by digging in their hole in the soil of the rice fields during the gestation period, from early March to late April. The animals were transported and acclimatized in the laboratory at Chulalongkorn University Forest and Research Station, Wiang Sa District, Nan Province, Thailand. The carapace width of each female crab was measured. Small plastic boxes (20×20×15 cm) were used to separate mothers from each other. The gestation season was recreated in the laboratory: The boxes were kept in dark, no food neither water was provided, only a water–saturated sponge was used to maintain high humidity in the boxes.

Daily egg sampling started the day after the specimens arrived at the laboratory at a fixed time (08:00AM). Twenty to forty eggs per mother were randomly removed from the abdominal pouch. The collected eggs were fixed in 70% ethanol and kept in 1.5 mL tubes at room temperature until the imaging. On the last day of sampling, all remaining eggs or juveniles were removed from the female pouch and included in order to appraise the total eggs for each clutch.

Fluorescence staining

For each stage, ten randomly collected eggs were first examined and the larval stages were digitally recorded using a stereomicroscope. The size of the eggs was measured according to the digital image. Until the 5^{th} day of development after spawning, all eggs were observed with their envelope. After the 5^{th} day of development, the embryo being located on the condensed egg yolk allowed the envelope to be removed, improving observations. The embryos were rehydrated in 50% ethanol for 30 min, then in 25% ethanol for 30 min and finally in phosphate buffered saline (PBS) for 30 min. The rehydrated eggs were fixed in 4% paraformaldehyde for 30 min then stained with 0.001% TRITC (tetramethylrhodamine isothiocyanate, Sigma Aldrich) for one hour to deliver fluorescence in the embryo.

Confocal microscope observation

The samples were analyzed with a Leica TCS–SPE confocal laser microscope (Montpellier RIO Imaging platform, France) to acquire three–dimensional data represented by stacks of images with a 1 µm step between each image (4

frames/image) for acquisition of about 200 µm thick. The staining and confocal observation were reproduced three times for each studied stage.

RESULTS

Rice field crabs were able to produce egg clutches ranging from 300 to 600 eggs (N = 30). Egg size varied among individuals, with an average diameter of about 1.34±0.27 mm (N = 120). The appearance of the egg was ball–shaped and yellowish in color, indicating an extreme quality of egg yolk and classifying it as a polylecithal type egg.

Pre-organogenesis

Stage 1 egg–cleavage (within 1 day of incubation; Fig. 6.2A)

The day after the night when the ovigerous crabs released their eggs, a pattern of superficial cleavage was noted in *E. nani* egg. At that time, many potential embryonic cells migrated under the shell membrane, indicating a late superficial cleavage (an earlier stage could not be isolated in this study). The cleavage did not show cytokinesis, thus giving several nuclei within the cytoplasm that can be considered as energids (Scholtz and Wolff, 2013). Thus, the energids correspond to the potential embryonic cells in this study. The egg yolk is fragmented into very fine droplets.

Stage 2 egg–blastula (At 2 days of incubation; Fig. 6.2B)

The obvious presence of the blastopore on one side of the edge of the egg certifies the blastula stage. Potential embryonic cells have begun to group together delimiting different cell groups. The cytosol started to be isolated under the shell membrane. The egg yolk is divided in slightly larger droplets.

Stage 3 initial egg–gastrula (At 3 to 4 days of incubation; Fig. 6.2C)

Individual cell membranes form at that stage. The first signs of a papilla positioned in the lateral–ventral position is observed (animal pole). It can be assumed that the cells of the blastula of the previous stage epibolically migrated from the surface of the egg to animal pole.

Stage 4 egg–gastrula (At 4 days of incubation; Fig. 6.3A and 6.3B)

The invagination of embryonic cells in the surrounding yolk forms a V–shaped papilla of cells. Drafts of the main embryonic cell groups (ocular, thoracic–abdominal and cephalic), all located ventrally, can already be seen. The labrum region, or the Hensen node or anterior primitive node, is located at the center of the invagination and the caudal region is connected to the primitive line resulting from the invagination. A few hours after the end of the egg–gastrula stage, the embryo has a protonauplius aspect (Fig. 6.4A).

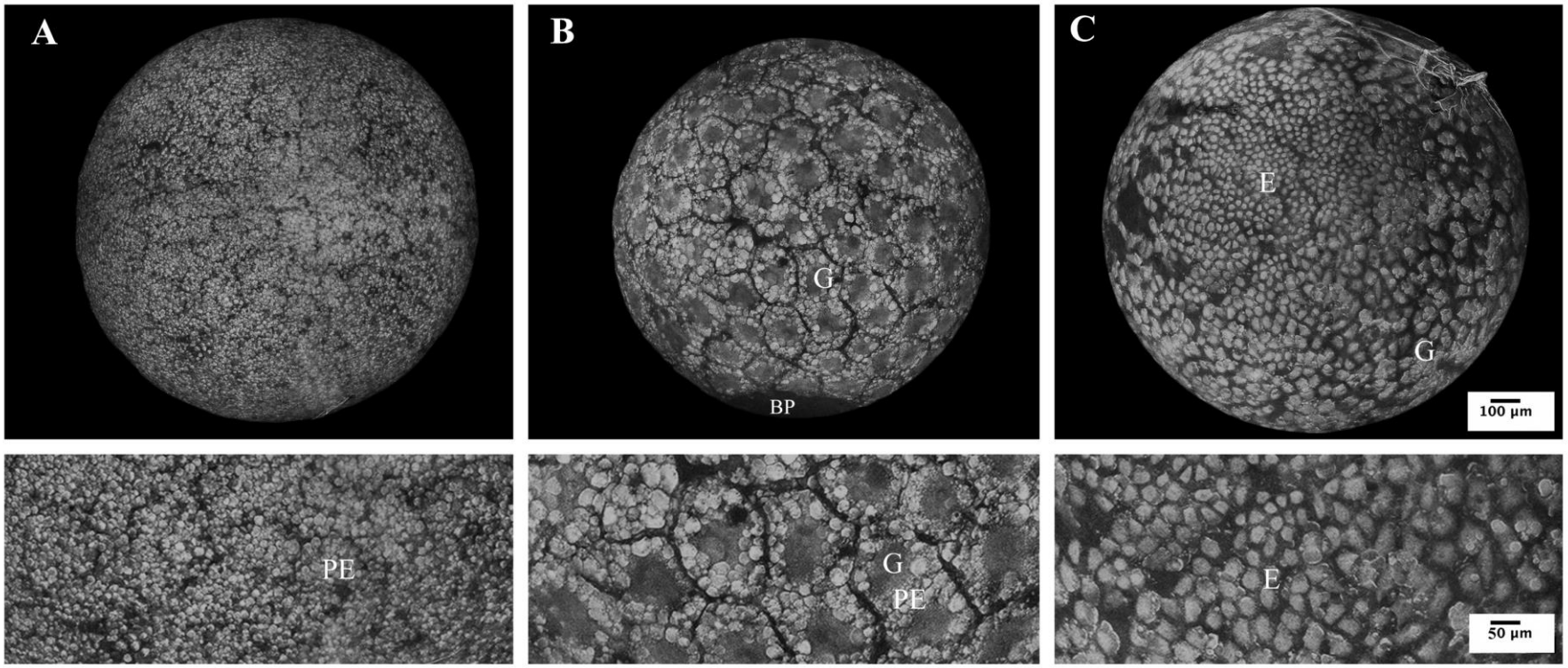


Figure 6.2 Confocal microscopic photographs representing *E. nani* embryos at pre–organogenesis stages part 1. A) egg–cleavage; B) egg–blastula; C) initial gastrula. The upper images show the whole eggs while the lower ones show a double magnification of the upper images. BP: blastopore E: embryonic cells; G: grouping of potential embryonic cell*;* PE: potential embryonic cells.

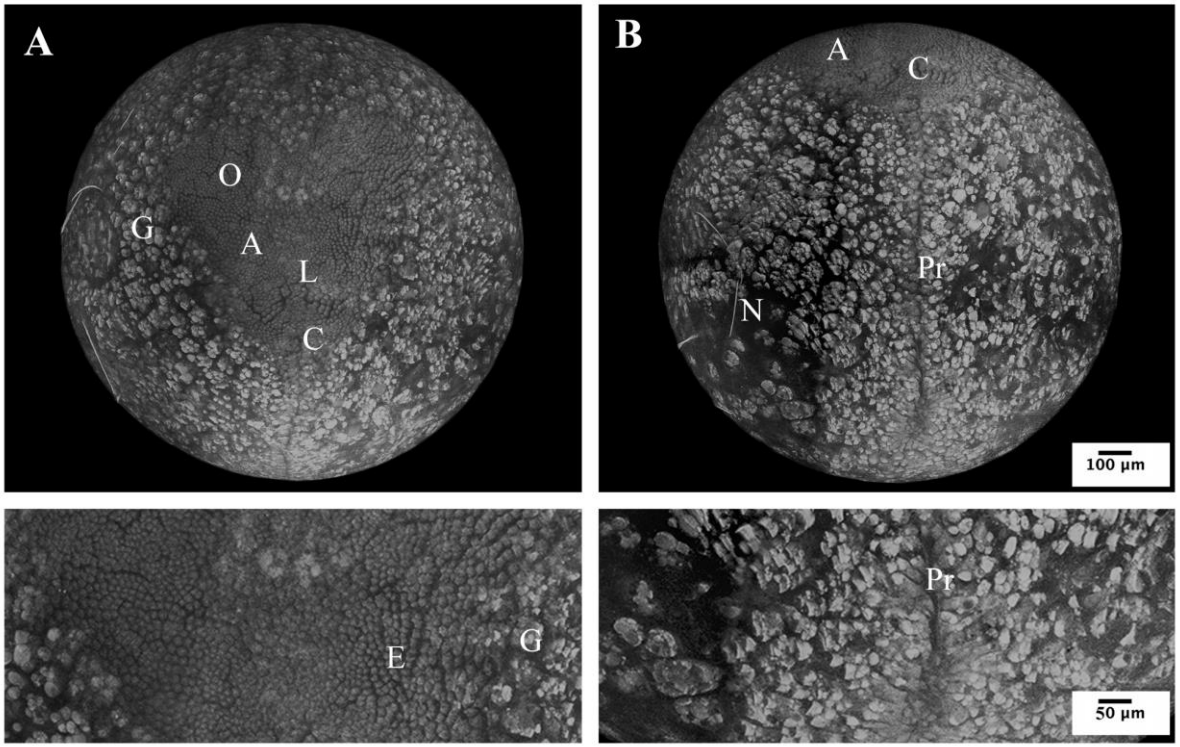


Figure 6.3 Confocal microscopic photographs representing *E. nani* embryos at pre–organogenesis stages part 2. A) gastrula with head view; B) gastrula with tail view. The upper images show the whole eggs while the lower ones show a double magnification of the upper images. A: antenna region; C: caudal papilla; E: embryonic cells; G: grouping of potential embryonic cell; L: labrum region; N: space without embryonic cell; O: optic region; Pr: primitive streak.

Organogenesis

Stage 5 egg–protonauplius (At 4–5 days of incubation; Fig. 6.4A)

The protonauplius is characterized by a rudimentary caudal papilla. This stage corresponds to the transition between pre–organogenesis to organogenesis stages. On either side of the invagination zone, an accumulation of cells becomes visible, so that the embryo is clearly identifiable on the surface of the yolk. From this stage, the egg membrane was removed to obtain better images. The rudiments of paired plates are increasing in relative size, due to cell proliferation, and unite to form an unpaired structure symmetrical to the longitudinal axis of the embryo (Fig. 6.4A).

Stage 6 egg–nauplius (At 5 days of incubation; Fig. 6.4B)

Thereafter, all the characters of the egg–nauplius stage are visible with the appearance of the first appendage primordia: a pair of buds of antennules, antenna and mandibles. This cluster corresponds to the first group of appendages of the head, specific to the embryonic development of crustaceans (Scholtz, 2000). The cells of the labrum region concentrate resulting in a labrum bud. Tiny structures positioned behind the ocular lobes are observed growing in the cephalic primordia. The mass of egg yolk moved and concentrated in the center of the egg, leaving space for further larval development.

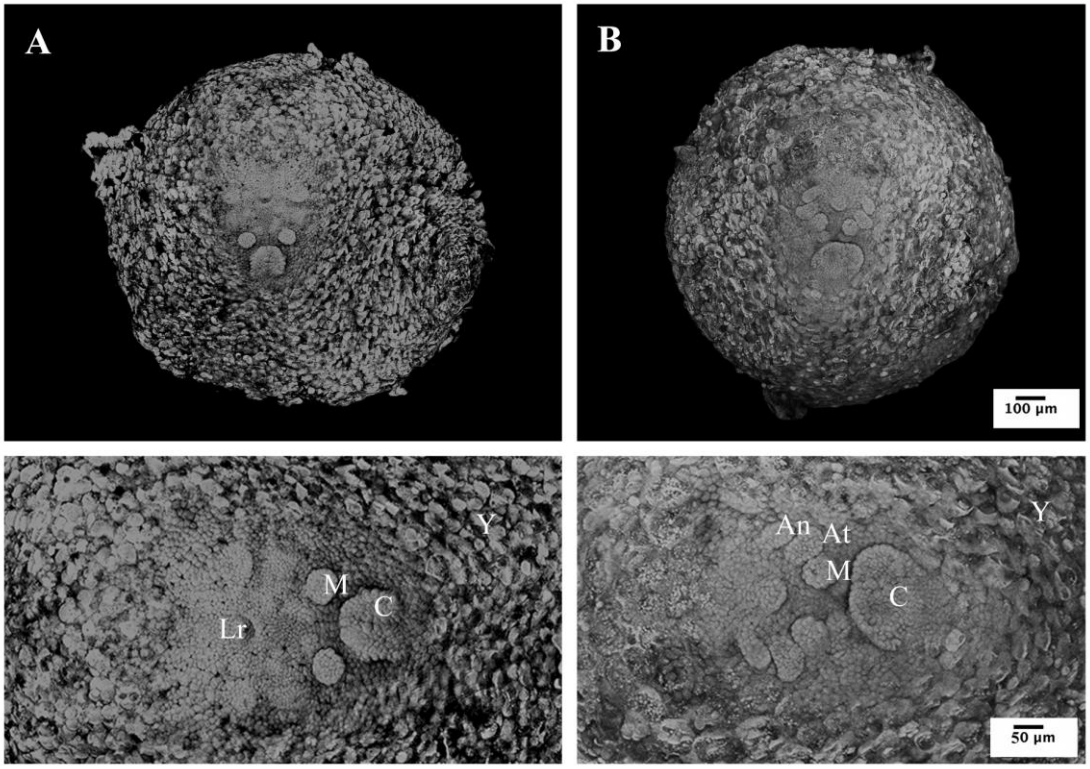


Figure 6.4 Confocal microscopic photographs representing *E.nani* embryos at organogenesis part 1. A) egg–protonauplius; B) egg–nauplius. The upper images show the whole eggs while the lower ones show a double magnification of the upper images. An: antennule appendage; At: antenna appendage; C: caudal papilla; Lr: labrum; M: mandibular appendage; Y: yolk.

Stage 7 egg–meganauplius (At 6 days of incubation; Fig. 6.5A)

The rudimentary optical lobes enlarge and diverge from each other. The thoracic appendages and segments are now visible. In addition to the paired naupliar appendages, the branchiostegal appendages, i.e. the first and second maxillae and the three maxillipeds, appear. Beside the branchiostegal appendages, a pair of branchiostegal folds are visible. The caudal papilla is segmented and presents the terminal duct of the intestine with the anus at its tip. Its folding covers part of the anterior region up to the level of the second maxillae.

Stage 8 early egg–zoea like (At 7 days of incubation; Fig. 6.5B)

The appendages are much larger, and the branchiostegal ones begin to duplicate the second branchiostegal buds. Branchiostegal folds grow on both sides to the posterior part of the embryo. The pairs of appendages of the legs split out from the caudal papilla.

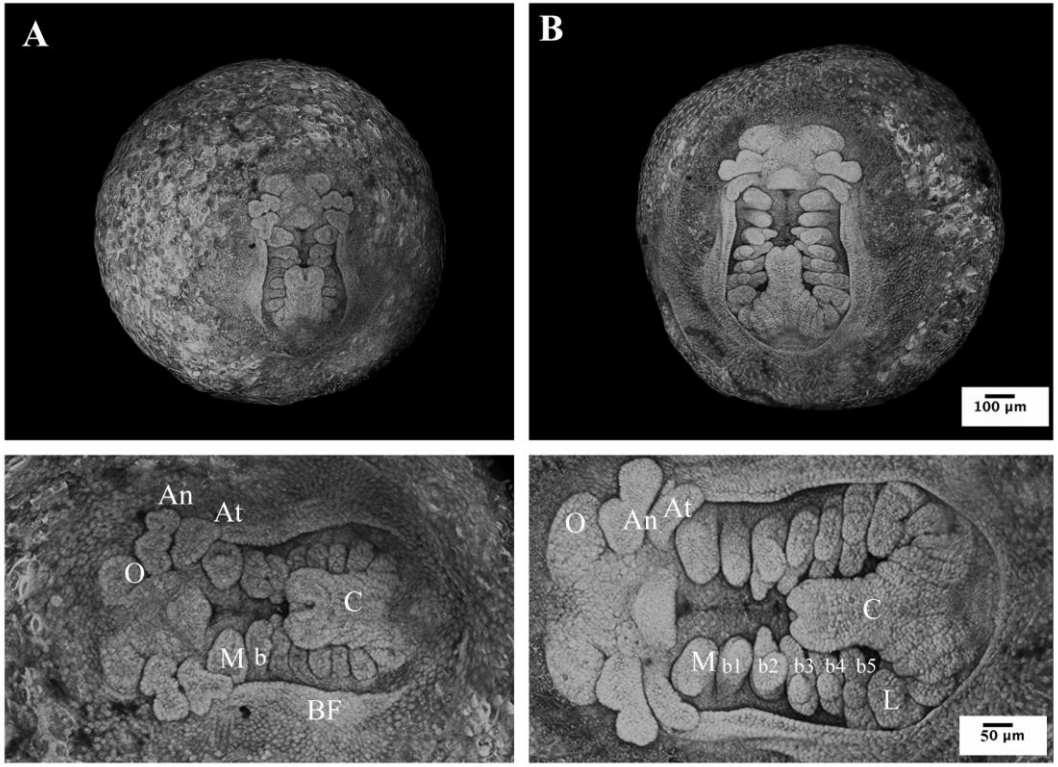


Figure 6.5 Confocal microscopic photographs representing *E.nani* embryos at organogenesis part 2. A) egg–metanauplius; B) egg–early zoea. The upper images show the whole eggs while the lower ones show a double magnification of the upper images. An: antennule appendage; At: antenna appendage; b1–5: branchiostegal appendages; BF: branchiostegal fold; C: caudal papilla; L: pereiopod or walking leg; Lr: labrum M: mandibular appendage; O: optic lobe.

Stage 9 late egg– zoea like (At 7–8 days of incubation; Figs. 6.6A-E)

The optical lobes reveal the eyestalk character (Fig. 6.6B). After the separation of the last pair of leg appendages from the caudal pillar, the rest of the caudal pillar appears as the true abdominal plate. The branchiostegal appendages are fully duplicated, and the 10 pairs can be considered as the final exopods. The rudimentary shell began to develop as embryonic cells migrated to the posterior part of the embryo and expanded from both sides of the branchiostegal folds.

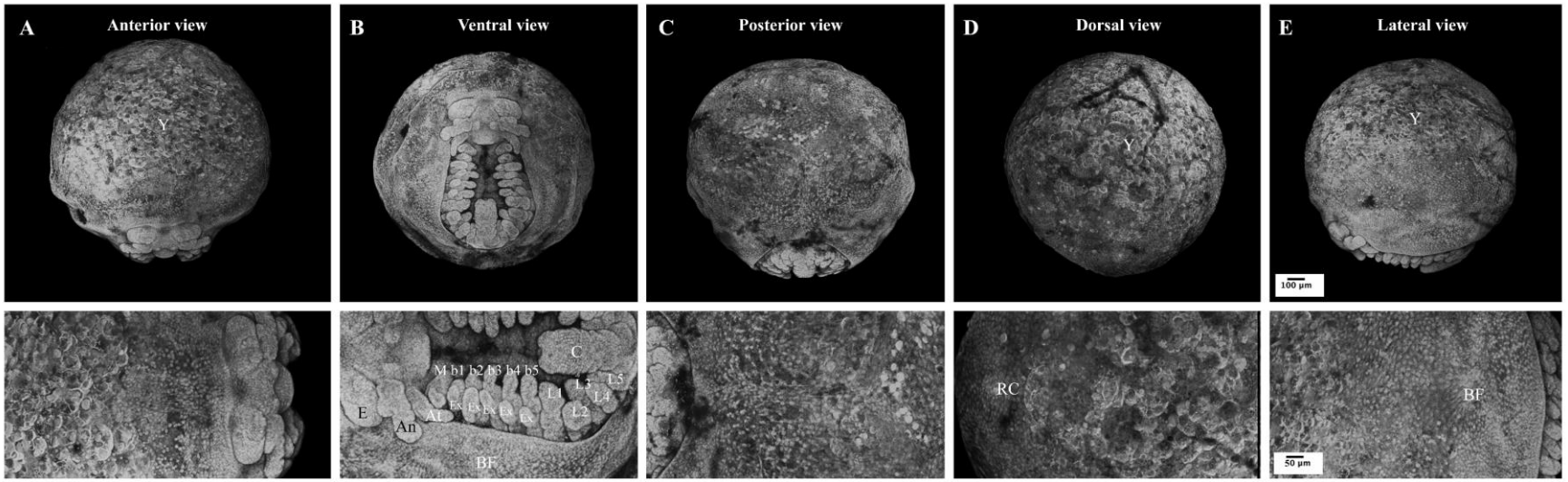


Figure 6.6 Confocal microscopic photographs representing *E.nani* embryos at organogenesis part 3. A–E) egg–late zoea. The upper images show the whole eggs (scale bars = 100 µm) while the lower ones show a double magnification of the upper images (scale bars = 50 µm). A: abdomen; An: antennule; At: antenna; b1–5: branchiostegal appendages; BF: branchiostegal fold; C: caudal papilla; E: rudimentary eyes; Ex: exopod–like; L1–5: pereiopod or walking leg; M: mandible; RC: rudimentary carapace; T: telson; Y: yolk.

Stage 10 early egg–megalopa like (At 8 days of incubation; Figs. 6.7A-E)

The antennules and antenna, the mandibles and maxillae become relatively smaller than the posterior thoracic limbs, which develop at a faster rate. The folding of the caudal papilla now only covers the part of the anterior region where the future legs are located (Fig. 6.7B). The major event at this stage is the formation of the shell. The dorsal view of the egg clearly shows the dorsal shell line and the rudimentary shell plates on the left and right and posterior sides (Fig. 6.7D).

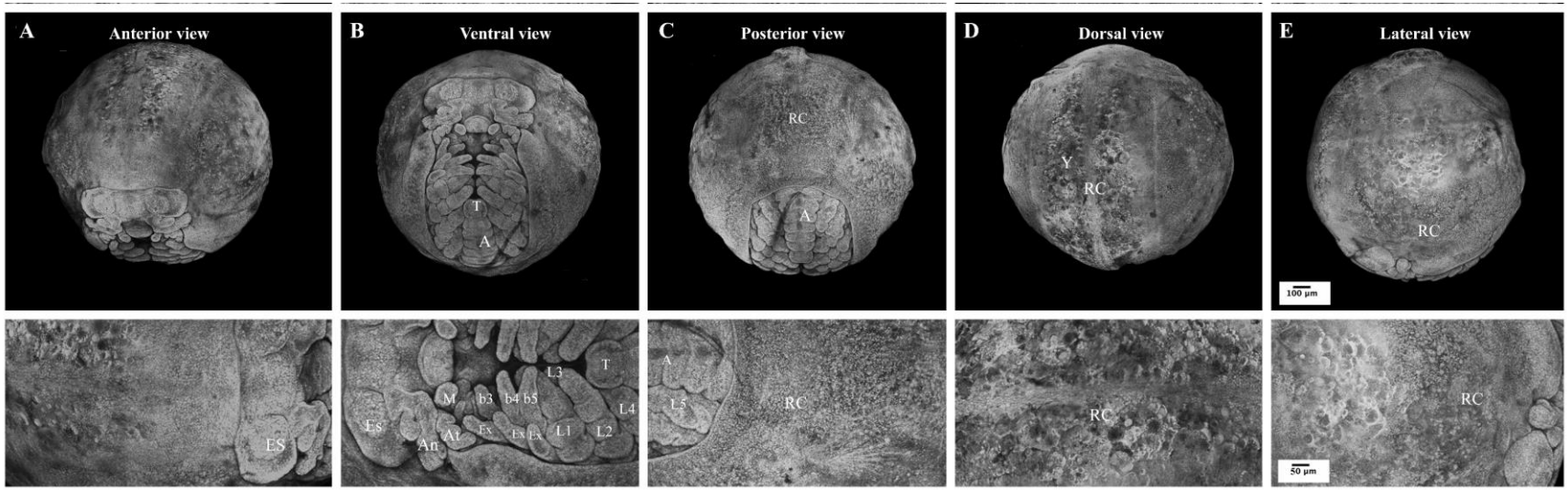


Figure 6.7 Confocal microscopic photographs representing *E.nani* embryos at organogenesis part 4. A–E) egg–early megalopa. The upper images show the whole eggs (scale bars = 100 µm) while the lower ones show a double magnification of the upper images (scale bars = 50 µm). A: abdomen; An: antennule; At: antenna; b3–5: branchiostegal appendages; ES: eyestalk; Ex: exopod–like; L1–5: pereiopod or walking leg; M: mandible; RC: rudimentary carapace; T: telson; Y: yolk.

Stage 11 egg–megalopa like (At 9 days of incubation; Figs. 6.8A-E)

The compound eyes are now pigmented. The distinct plates of the shell have fused to form a single shell plate that surrounds most of the cephalo–thoracic viscera. Some duplicated branchiostegal buds disappear, some of them even fold back. The first walking–legs have grown, and their tips display a scissor–like shape.

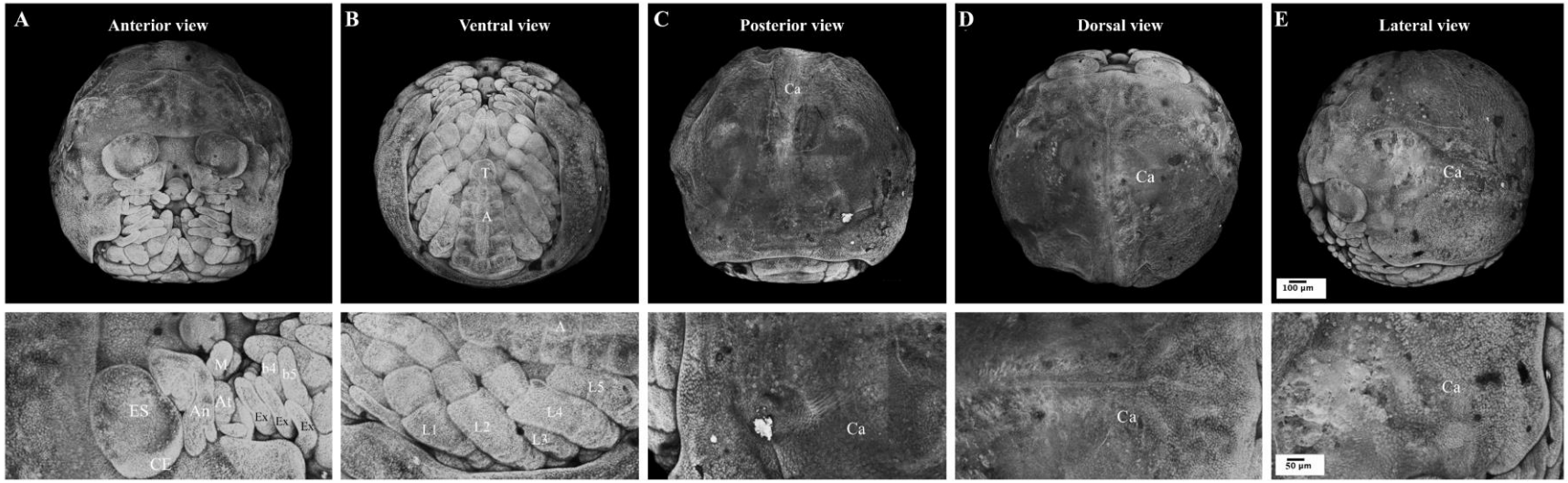


Figure 6.8 Confocal microscopic photographs representing *E.nani* embryos at organogenesis part 5. A–E) egg–megalopa. The upper images show the whole eggs (scale bars = 100 µm) while the lower ones show a double magnification of the upper images (scale bars = 50 µm). A: abdomen; An: antennule; At: antenna; b4–5: branchiostegal appendages; Ca: carapace; CE: compound; ES: eyestalk; Ex: exopod–like; L1–5: pereiopod or walking leg; M: mandible; T: telson.

Stage 12 late egg–megalopa like (At 10–11 days of incubation; Figs. 6.9A-E, 6.10A-E)

These stages occur about 2–3 days before hatching. Eyestalks, antennules, antennae, and mandibles are fully developed. All the organs are fully formed and ready to perform their function. The main event at this stage is the increase in size of the embryo.

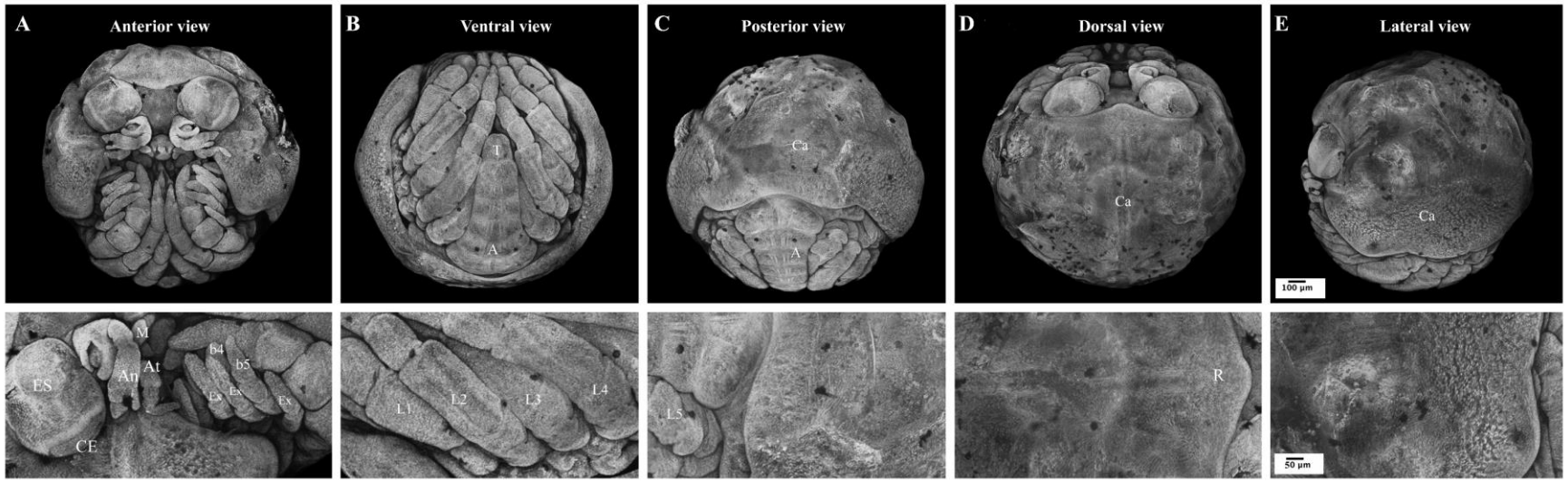


Figure 6.9 Confocal microscopic photographs representing *E.nani* embryos at organogenesis part 6. A–E) egg–late megalopa I. The upper images show the whole eggs (scale bars = 100 µm) while the lower ones show a double magnification of the upper images (scale bars = 50 µm). A: abdomen; An: antennule; At: antenna; b4–5: branchiostegal appendages; Ca: carapace; CE: compound; ES: eyestalk; Ex: exopod–like; L1–5: pereiopod or walking leg; M: mandible; R: rostrum; T: telson.

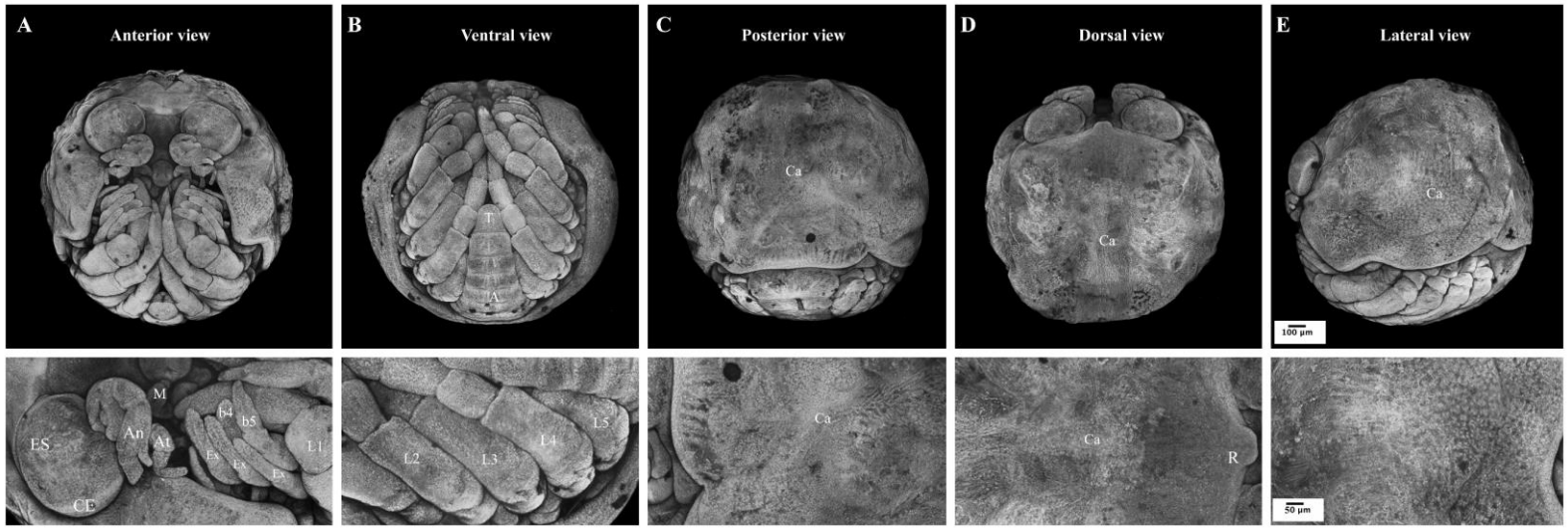


Figure 6.10 Confocal microscopic photographs representing *E.nani* embryos at organogenesis part 7. A–E) egg–late megalopa II. The upper images show the whole eggs (scale bars = 100 µm) while the lower ones show a double magnification of the upper images (scale bars = 50 µm). A: abdomen; An: antennule; At: antenna; b4–5: branchiostegal appendages; Ca: carapace; CE: compound; ES: eyestalk; Ex: exopod–like; L1–5: pereiopod or walking leg; M: mandible; R: rostrum; T: telson.

Hatching stage

Stage 13 hatching megalopa like (At 12 days of incubation; Figs. 6.11A-E)

At hatching, the cuticle is fully formed. As a result, TRITC staining could not reach the embryo. Stereomicroscope images were used to replace confocal microscope ones. The overview characters of the hatching stage are very similar to those of the previous stage. The shell is covered with a translucent membrane, which is supposed to protect the soft shell. At hatching, the average carapace size of the megalopa was 1.375±0.093 cm wide and 1.357±0.141 cm long, and the cephalothorax was 1.401±0.150 cm deep (N = 24).

Stage 14 juvenile crab (At 12 days of incubation; Figs. 6.11F-J)

A few hours after hatching, the megalopa larvae metamorphosed into a juvenile crab. The shape of the juvenile carapace has widened compared to that of the megalopa carapace due to the absence of the physical constraints generated by egg membrane. The five pairs of branchiostegal appendages were transformed into five pairs of mouthparts. The first pair of walking legs develops like a pair of chelae. The average carapace size of the first juvenile was 1.422±0.176 cm wide and 1.361±0.126 cm long, and that of the cephalothorax depth was 1.151±0.158 cm (N = 24).

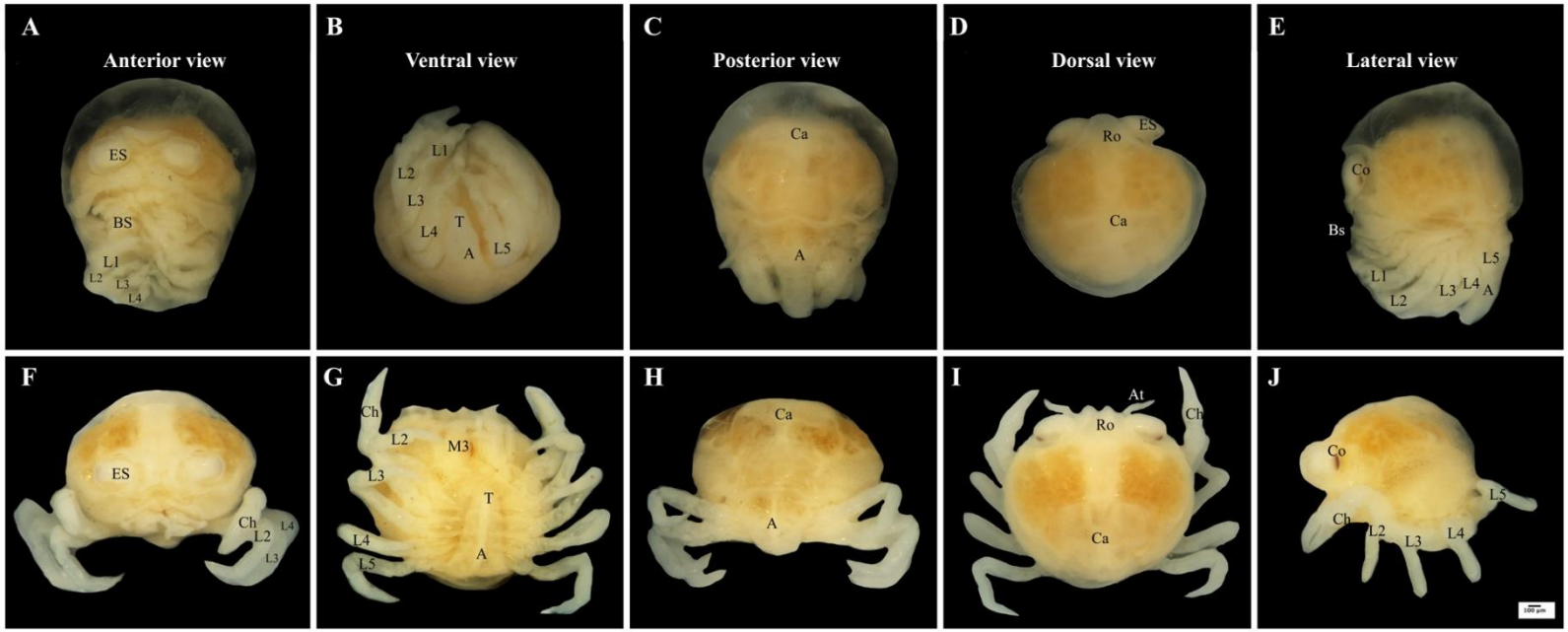


Figure 6.11 Stereomicroscopic photographs representing *E. nani* at hatching stages. A-E): hatching–megalopa; F-J: first instar juvenile. A: abdomen; At: antenna appendage; Bs: branchiostegal appendages; Ca: carapace; Ch: chela; Co: compound eye; ES: eyestalk; M3: third maxilliped; L1–5: pereiopod or walking leg; Ro: rostrum; T: telson.

Table 6.1 Comparison of morphological features of appendages/characters at embryonic/larval stages of *Esanthelphusa nani* [Gecarinucidae] from the present study; *Sinopotamon yangtsekiense* [Potamidedae] by Wu et al. (2010); *Potamon edulis* [Potamidedae] by Pace et al. (1976); *Scyra acutifrons* [Epialtidae] by Oh and Sook Ko (2010); *Nanocassiope melanodactyla* [Xanthothidae] by Dornelas et al. (2004); *Sesarma curacaoense* [Sesarmidae] by Anger et al. (1995); *Brachynotus sexdentatus* [Varunidae] by Cuesta et al. (2000); *Geograpsus lividus* [Grapsidae] by Cuesta et al. (2011); and *Ranina ranina* [Raninidae] by Sakai (1971)

Section/Subsection	Eubrachyura/Heterotremata					Eubrachyura/Thoracotremata			Raninoida
Family	Gecarinucidae	Potamidedae	Potamidedae	Epialtidae	Xanthothidae	Sesarmidae	Varunidae	Grapsidae	Raninidae
Species	*Esanthelphusa nani*	*Sinopotamon yangtsekiense*	*Potamon edulis*	*Scyra acutifrons*	*Nanocassiope melanodactyla*	*Sesarma curacaoense*	*Brachynotus sexdentatus*	*Geograpsus lividus*	*Ranina ranina*
Habitats	Rice field, freshwater	River, freshwater	Streams, freshwater	Marine	Marine	Mangrove	Coastal to marine	Marine	Marine
Type of development	Direct development	Direct development	Direct development	Abbreviated development	Extended development	Abbreviated development	Extended development	Extended development	Extended development
Hatching stage	Megalopa	Juvenile	Juvenile	Zoea	Zoea	Zoea	Zoea	Zoea	Zoea
Total period of embryonic/larva development	12 days in eggs	77 days in eggs	46–47 days in eggs	Not available	30 days after hatching	17–20 days after hatching	17–22 days after hatching	60 days after hatching	Not available
Appendages/Characters	Onset at stages	Onset at stages	Onset at stages	Onset at stages	Onset at stages	Onset at stages	Onset at stages	Onset at stages	Onset at stages
1st ANTENNA	egg nauplius	egg nauplius	egg nauplius	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea1,Zoea2 (?)	Zoea1–Zoea5 (?)	Zoea1–Zoea8 (?)
2nd ANTENNA	egg nauplius	egg nauplius	egg nauplius	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea1,Zoea2 (?)	Zoea1–Zoea5 (?)	Zoea1–Zoea8 (?)
MANDIBLE	egg nauplius	egg nauplius	egg nauplius	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea1,Zoea2 (?)	Zoea1–Zoea5 (?)	Zoea1–Zoea8 (?)
MAXILLULE	egg metanauplius	egg zoea like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea1,Zoea2 (?)	Zoea1–Zoea5 (?)	Zoea1–Zoea8 (?)
MAXILLA	egg metanauplius	egg zoea like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea1,Zoea2 (?)	Zoea1–Zoea5 (?)	Zoea1–Zoea8 (?)
1st MAXILLIPED	egg metanauplius	egg zoea like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea1,Zoea2 (?)	Zoea1–Zoea5 (?)	Zoea1–Zoea8 (?)
2nd MAXILLIPED	egg metanauplius	egg zoea like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea1,Zoea2 (?)	Zoea1–Zoea5 (?)	Zoea1–Zoea8 (?)
3rd MAXILLIPED ***	egg metanauplius	egg megalop like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea2,Zoea3,Zoea4	Zoea1,Zoea2 (?)	Zoea3,Zoea4,Zoea5	Zoea6,Zoea7,Zoea8	Zoea1–Zoea8 (?)
1st PEREIOPOD ***	egg zoea like	egg megalop like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea2,Zoea3,Zoea4	Zoea1,Zoea2 (?)	Zoea3,Zoea4,Zoea5	Zoea6,Zoea7,Zoea8	Zoea1–Zoea8 (?)
2nd PEREIOPOD	egg zoea like	egg megalop like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea3,Zoea4	Zoea1,Zoea2 (?)	Zoea3,Zoea4,Zoea5	Zoea6,Zoea7,Zoea8	Zoea1–Zoea8 (?)
3rd PEREIOPOD	egg zoea like	egg megalop like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea3,Zoea4	Zoea1,Zoea2 (?)	Zoea3,Zoea4,Zoea5	Zoea6,Zoea7,Zoea8	Zoea1–Zoea8 (?)
4th PEREIOPOD	egg zoea like	egg megalop like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea3,Zoea4	Zoea1,Zoea2 (?)	Zoea3,Zoea4,Zoea5	Zoea6,Zoea7,Zoea8	Zoea1–Zoea8 (?)
5th PEREIOPOD	egg zoea like	egg megalop like	egg metanauplius	Zoea1,Zoea2 (?)	Zoea3,Zoea4	Zoea1,Zoea2 (?)	Zoea3,Zoea4,Zoea5	Zoea6,Zoea7,Zoea8	Zoea1–Zoea8 (?)
CARAPACE	egg megalop like	egg megalop like	egg megalop like	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea3,Zoea4,Zoea5	Zoea6,Zoea7,Zoea8	Zoea1–Zoea8 (?)
Segmented ABDOMEN	egg megalop like	egg megalop like	egg zoea like	Zoea1,Zoea2 (?)	Zoea1 (?)	Zoea1,Zoea2 (?)	Zoea1,Zoea2 (?)	Zoea1–Zoea5 (?)	Zoea1–Zoea8 (?)
Bent/Fused TELSON	egg zoea like	egg megalop like	egg megalop like	Megalopa	Megalopa	Megalopa	Megalopa	Megalopa	Never

Note: – The order of presentation is based on the phylogenetic relationships among Brachyura family according to Tsang et al. (2014)
– *** describe the important transitions in the appearance of appendages.
– the interrogation mark (?) indicates the possibility of already having appendages in the eggs.

DISCUSSION & CONCLUSION

This study is the first one using confocal microscopy to document crab development. In the case of primary freshwater crab, this technology allowed to deal with the insufficient resolution offered by stereomicroscope. It was particularly relevant for the part of the development occurring within the egg since primary freshwater crabs have direct development contrary to other crabs for which early stages are free and can be easily observed. The use of confocal microscopy was facilitated by the development of the crab which follows a superficial cleavage development, exposing the embryo close to the surface of the egg.

Table 6.1 shows comparison among crabs for which the embryonic development is known. This study is the first one looking at the ontogenetic sequences of primary freshwater crabs of the Gecarinucidae family. It further documents the development of primary freshwater crabs at a broader taxonomic scale. Former studies have focused on primary freshwater crabs of the Potamidae family (Pace et al., 1976; Wu et al., 2010). Among the crabs studied, the development of *E. nani* produced hatchlings that are adult like in shape within a much shorter development (12 days) by comparison to the two other ones that were documented (77 days for *S. yangtsekiense* and 46–47 days for *Potamon edulis*). This shortened development, however, was not accompanied by major changes in organogenesis, showing that this pattern was probably shared in all primary freshwater crabs of the clade containing Potamidae, Potamonautidae, Gecarcinucidae since this group appears monophyletic in the study of Tsang et al. (2014).

As in other primary freshwater crabs, the development is direct. Therefore, the crab does not have free nauplius, zoea, or free early megalopa stages. However,

similarities were found with those stages based on the developmental sequences of appendages as in the work of Pace et al. (1976) and Wu et al. (2010).

The development of the embryo of *E. nani* is superficial and can be classified in the superficial cleavage model. It is similar with the development of most land arthropods such as, for instances, the land isopod *Porcellio scaber* (Wolff, 2009) or the land anomuran *Aegla platensis* (Lizardo-Daudt and Bond-Buckup, 2003). Cleavage is limited by a massive yolk mass in the center that confines mobile cleavage cells to the cytoplasmic edge of the egg (Schetelig et al., 2007; Eriksson and Tait, 2012). Furthermore, there are no partitions and no cell membranes delimiting cells in the early development as for the coenocytic blastoderm of some other terrestrial arthropods (Fig. 6.2A). By contrast, early total cleavage can be present in secondary freshwater crabs as in the mitten crab *Eriocheir japonica* (Varunidae) or in marine crabs such as the fiddler crabs *Uca lacteal* (Ocypodidae) (Kobayashi and Matsuura, 1995; Yamaguchi, 2001). Interestingly the modes of cleavage do not necessarily express a strong relationship with arthropod phylogeny (Peterson and Eernisse, 2001; Scholtz and Wolff, 2013), but might be related to yolk mass quantity, which itself may relate with the ecology. Further exploration at the infra–order or suborder level would be interesting in that respect. Freshwater crabs of the families Trichodactylidae and Pseudothelphusidae would be particularly interesting to document since they represent independent evolution to the freshwater environment (Fig. 6.1).

Early in the development, the “egg nauplius” stage showed obvious naupliar appendages with no segmentation (antennule, antenna and mandible appendages that are associated with neurology and myology). This part of development is preserved

among Eumalacostraca (Scholtz, 2000; Vogt, 2013). but also present outside of the Eumalacostraca since it is also reported in Pleocyemata, a member of decapods (Jirikowski et al., 2013).

At later stages, unlike the marine crab, post–nauplius stages of the primary freshwater crab form within the eggs. The transition between larval stages of the primary freshwater crab seems to be more continuous (without molting) and more difficult to categorize than those of the marine crab. In general, free–living zoae stages are characterized based on characters of maxillipeds, pereiopods and pleopods (da Silva et al., 2012; Magalhães et al., 2017). When compared with marine crabs (for instance Xanthoidea in (Clark, 2005), Table 6.1), zoea stages show differences in terms of appearance of setae, endopod and exopod on maxillipeds. Likely, these differences come from the fact that this appendage is not yet used for feeding or moving as in free–living zoea stage (Epelbaum and Borisov, 2006). Pleopods of *E. nani* were not evidenced but might be hidden by their growth under the abdominal plate as the histologic section of *Potamon edulis* in zoea stage presented up to 8 thorax somites which relate to appendages under abdomen (Pace et al., 1976). Using of pereiopods should be noted as a suitable external character of larva for extricating stages of egg–zoae and egg–megalopa stages in crustacean with direct development such as the primary freshwater crab. The onset of appendage development differs greatly among crab lineages (Table 6.1). *E. nani* seems to be intermediary in terms of onset for appendage apparition by comparison to body shape development among documented freshwater crabs. It is delayed by comparison to *P. edulis* but accelerated by comparison to *S. yangtsekiensise*. This difference does not seem to be associated with the rapid development of *E. nani* since it develops faster than the two potamid

species. Further studies might be necessary to see whether development timings in Gecarinucidae is as variable as in Potamidae. It is eventually speculated that this disparity in freshwater crabs may greatly depend on the variety of terrestrial environment and to their degree of terrestrialization.

Interestingly, it is noticed that development timing is not necessary linked to the ecological categories (primary freshwater, secondary freshwater and marine crabs). Also, direct development is a character of all freshwater crabs, the abbreviation of larval stages does not only occur in secondary freshwater crabs but in some marine crabs (e.g. *S. acutifrons* in Heterotremata). This convergent pattern might not be only related to main habitat ecology (Vogt, 2013), but to other ecological characteristics and likely life traits history. By looking more in details in the sequence of appendage apparition during abbreviated development, it is, however, possible to define characters that are related to phylogeny and others to habitat. In particular, appendage shape and development may be affected by the environment where the larva evolved. Obviously, pereiopod and pleopod appendages of marine larva are different in shape from terrestrial species. Setae in free living larva of marine crab tends to be more numerous than the one developing in eggs, possibly due to the fact that this appendage is not yet used for feeding or moving as in free–living zoea stage (Epelbaum and Borisov, 2006).

Although the current study extended the knowledge about early development in primary freshwater crabs, it would be interesting to study other group of freshwater crabs to generalize this findings and to define further differences and similarities that could be phylogeny or ecology dependent in order to complete the comparison table. In particular, primary freshwater crabs are not monophyletic and this ecology might

have evolved at least three times in the infra–order Brachyura (Fig. 6.1). So far, the development has been described in three crabs of the clade containing Potamidae, Gecarcinucidae and Potamonautidae, but these groups may inherit their ecology and development from the common ancestor. Therefore, it would be interesting to compare differences and similarities with other freshwater clades such as Trichodactylidae or Pseudothelphusidae which are supposed to have independently evolved a primary freshwater morphology. Furthermore, it would be important to document the developmental sequence in marine sister groups to be able to partition out which characters might be related to ecology and which one might relate to phylogeny. It would also be interesting to further compare the development among primary freshwater crabs that show various degrees of terrestrialization and parental care to see how it might influence the morphology of the embryo at the different stages. Comparison with other groups could allow to understand convergent pattern in a more functional way. For instance, loss of free–living zoea stage is not unique to primary freshwater crabs but is known also in shrimps and is used to separate Palaemonidae and Atyidae (Jirikowski et al., 2013). Finally, documenting early stages of development in marine and secondary freshwater crabs within their eggs could improve the understanding of developmental timing, in particular in terms of appendage development.

CHAPTER VII

General Conclusion and Recommendations

Esanthephusa nani inhabits in the rice fields which are artificial wetlands that are subjected to be changed by agricultural activities throughout the year. This habitat is also extremely different from marine environment in terms of available space, resource and heterogeneity. In order to survive in this changing environment, the species evolved its physiology and development accordingly. By comparison to marine species, the freshwater crab has to evolve different strategies for water and mineral homeostasis (Augusto et al., 2007). Moreover, the crab had to evolve specific reproductive physiology to ensure the survival of offspring. *E. nani* is a good example of crab species to study changes in development and reproductive biology associated with the environmental shift from marine to freshwater and terrestrial environment. This study showed several traits in developmental and reproductive biology that could be associated with its ecology. On the other hand, some characters are shared with other crabs that are not necessarily primary freshwater and could be related to the phylogenetic history.

Male *E. nani* can produce the sperm throughout the year. However, there is a cyclical variation within the year. This variation may be related to a tradeoff between the resource to produce gametes and to release these gametes at the right time for mating with females. Gonadosomatic index (GSI) of male showed seasonal changes. Testicular activities were classified into different stages of testis based on the significant difference of GSI. These three phases are active testis, developing testis, and resting testis based on spermiogenesis activity. These 3 testicular phases could be

associated with the development of the reproductive endocrine organs such as mandibular organ (MO). Of particular importance, MO is indeed involved in hormonal secretion for stimulating the growth of testis and responds directly to environmental cues. It is also linked with the inhibiting hormone from eyestalk-X-organ-sinus-gland axis (Nagaraju and Borst, 2008). Its development could be controlled by resource availability (such as rainfall) and, therefore, could stimulate the development of gonads and increase of GSI. The hormone released is also associated with mating, since hormone could influence the male behavior so that they will look for mating with female instead of foraging or territory defense.

Lack of spermatophores in vas deferens (VDs) in *E. nani* is an unexpected discovery in this research. The production of spermatophores seems to be, indeed, the rule in crabs. However, data are still scanty to understand the distribution of this character according to the ecology and phylogeny. In marine crabs, spermatophore are usually observed and might help to transport massive sperm products through the vas deferens. Spermatophores are also observed in freshwater species such as *Sylviocarcinus pictus* (Trichodactylidae) (Silva et al. 2012). *E. nani* (Gecarcinucidae), however, was found to be similar to *Sundathelphusa philippina* (Gecarcinucidae) that does not produce spermatophores (Klaus et al., 2013). Spermatozoa are free in the seminal fluid contained in VDs. In addition, the spermatozoa are still free within the female spermatheca throughout the year (Fig. 7.1). Whether this change is related to different lifestyles and changes of reproductive strategy (*k* to *r*) must be further examined by more comparative studies considering the size of spermatophore, their absence and the number of offspring released by the female.

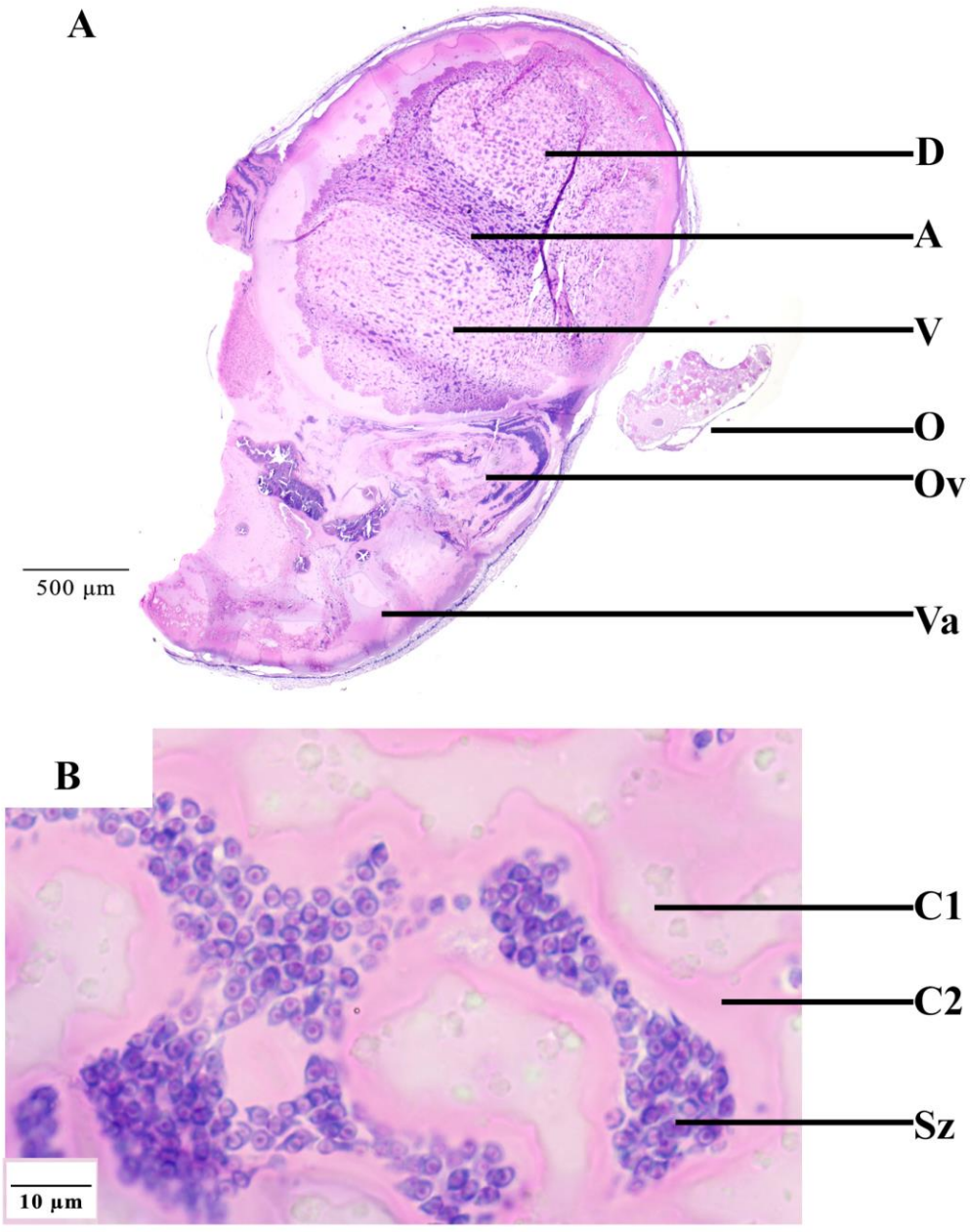


Figure 7.1 Histological observation of female *E. nani* spermatheca. A) Macrostructure of spermatheca consists of atrium (dorsal and ventral) and vagina. B) Mature spermatozoa deposited in spermatheca atrium. A: atrium; C1: secretion type 1; C2: secretion type 2; D: dorsal atrium; O: ovary; Ov: oviduct opening; Sz: spermatozoa; Va; vagina; V: ventral atrium.

While change in male reproduction was noticed, female *E. nani* also have evolved special strategies in terms of reproduction. In particular, they produce relatively low number of eggs (400-600 eggs), but the eggs are more fertile. This

number is very different from marine crab, *Portunus trituberculatus* (Portunidae), that can produce several millions of eggs (Hamasaki et al., 2006). The size of eggs was about 1.5 mm in freshwater crab, *E. nani*. This size is greater than the 0.1-0.2 mm of egg size in marine crab, *Scylla paramamosain* (Portunidae) (Islam et al., 2010). Moreover, cyclic change in ovarian structure during the year was found. The macroscopic examination of *E. nani* ovary showed a dramatic increase of ovarian size during the annual cycle from resting stage to mature ovarian stage. The ovarian color changed from white to orange which indicates yolk deposit during egg development. The histological examination of the female ovary showed how massive eggs could be derived from oogonia which developed and increased their size up to 30 times to become the mature oocyte. The mature oocyte cytoplasm was full of yolk droplets. As in other primary freshwater crabs, this large amount of yolk enables the crab to develop in the egg until reaching a much more mature stage than in marine species.

There are several peaks of GSI during the year for female *E. nani*. As expected, the cycle of GSI shows a tendency to synchronize with vitellogenin levels in hemolymph based on alkali–labile phosphate assay. In other freshwater crabs, *Sinopotamon henanense* (Potamidae), a single peak of GSI was observed within a year (Sun et al., 2018). The GSI of female *E. nani* is not following the pattern of GSI of the male *E. nani* and is therefore not directly related to wet season and resource availability. In female crab, at least one clutch of offspring could be produced during the wet season when the peak of GSI occurred. The female cycle showed that egg clutches were not released during estivation period or during the dry season. This means that juvenile crabs are released in the environment only when the environment is fertile enough so that they can properly continue their development. The female can

decide when to fertilize eggs and release them since she has spermatheca to store the spermatozoa produced by the male.

The development in *E. nani* is direct but also one of the faster known for the freshwater crabs. The direct development is related to the large size of the eggs, and the rich in egg yolk. The fast development allows *E. nani* to survive in a fast changing environment such as rice fields in northern Thailand where there is an alternation of wet and dry seasons. Comparing to the river crab, *Sinopotamon yangtsekiense* (Potamidae), which has 70 days of development (Wu et al., 2010), the stream crab, *Potamon fluviatile* (Potamidae), which has 40 days of development (Pace et al., 1976), the *E. nani* embryonic development (within 12 days) is the shortest period reported to date. This short development is not necessarily related to a decrease in maternal care. Indeed, *E. nani* juveniles are still attached and fed by the ovigerous female crab for at least two weeks in the wet season (based on a laboratory observation as shown in Fig 7.2). It is similar to the juvenile *Sylviocarcinus pictus* (Trichodactylidae) that stay on the ovigerous female crab for approximately 17 days (Sant'Anna et al., 2013). It is also observed that some ovigerous females could be found during the dry season. In that case, they might keep juveniles for a longer time (i.e. for a few months) during the estivation. This involves the large investment on maternal care in this period including feeding the juveniles as well as managing the moisture / water supply for the young.

These findings on the crab reproductive biology can have important implication for controlling (increase / decrease) population in the natural habitat in Nan province.

The direct development of *E. nani* is very fast (within 12 days). Those embryos completely develop and hatch as juveniles that feed on female abdominal pouch. Male *E. nani* can produce sperm throughout the year, while the female shows a cyclical variation of ovarian development within the year. Therefore, the female crab could be a better target for population management.

The female usually has sperm stock in the spermatheca, which means that their mating time is not related to zygotic production. Based on the current result of female reproductive cycle, the female crab can produce offspring at two possible times in one year: one in dry season and at least another one in wet season. To manage the population, the current study suggests that the suitable period is between the end of dry season (or an estivation period) toward the early wet season (June-August). In this period, the ovigerous crabs would come out of their borrows and search for water in the rice field. In the meantime, the rice farming activity also resume in this period so that the rice field is relatively empty without plant and weed. As the result, the ovigerous can be easily caught by hand. When one ovigerous is eliminated, at least three hundred juvenile crabs are also removed. Since the female crabs could potentially spawn the second clutch of offsprings during wet season, eliminating the ovigerous crab in the early wet season also play role as preventing the second spawning.

The ovigerous crabs that are caught in the early wet season can also be used as a source of crab stock in farm system. Each ovigerous crab can provide at least 300 juveniles for culture. The juvenile can be raised in captivity in farm system at the begin of the active season. With several months to grow from juvenile to adult stage before the estivation begins in the next dry season, these juveniles will become large

size crabs with substantial hepatopancreas deposit and can be sold at an excellent price.

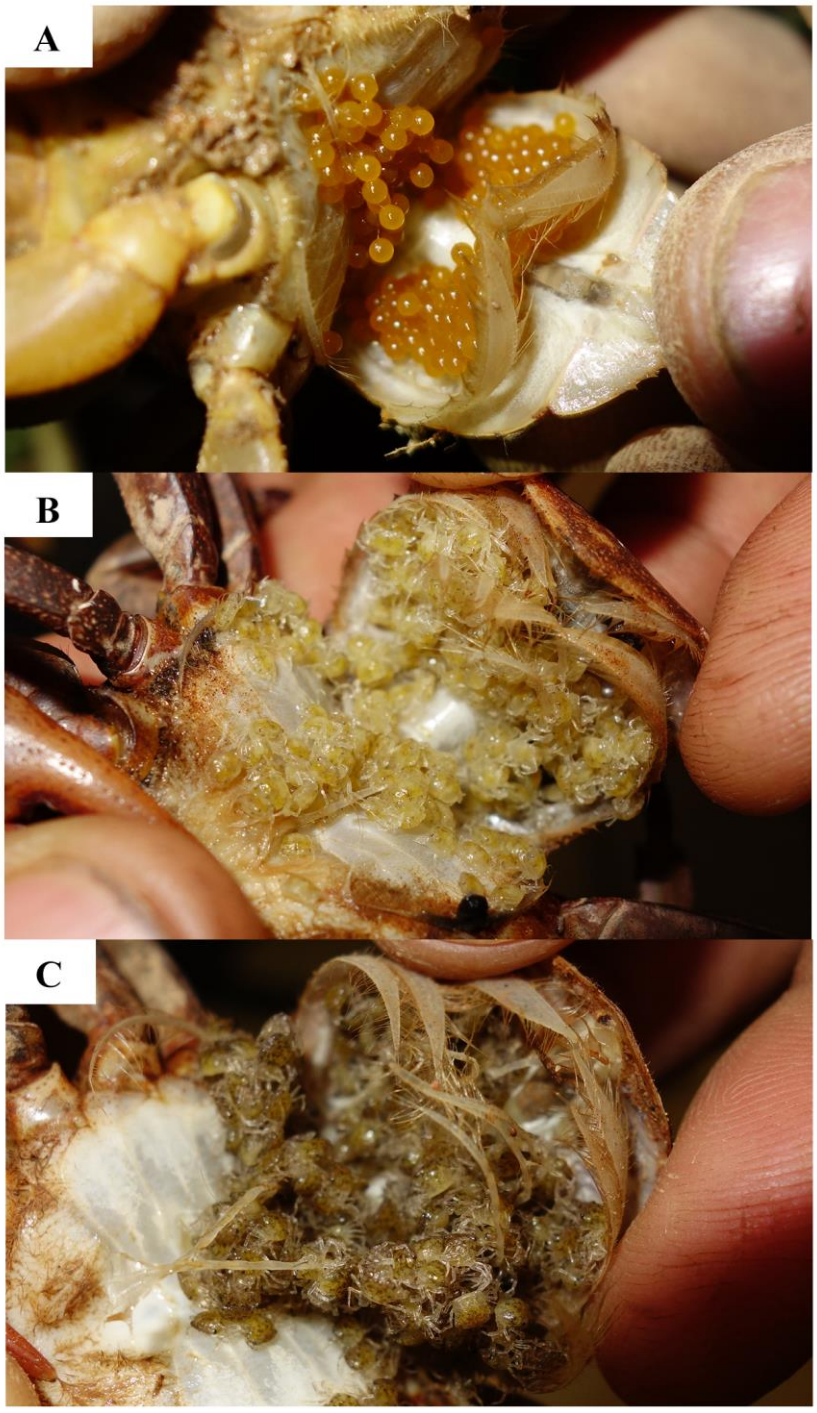


Figure 7.2 Brood care of ovigerous crab *E. nani*; A) The embryo in eggs attracted to ovigerous pouch; B) A few days old juveniles in the ovigerous pouch; C) Two weeks old juveniles in the ovigerous pouch.

Recommendations

This study opens possibilities to explore further patterns or questions as follows.

1. It would be good to examine variation in male crab seminal fluid quality in vas deferens because it might be related with sperm quality and mating cycle.
2. Mandibular organ as well as other endocrine organs that are associated with gonad development (see the pathway in Chapter II) should be investigated to produce a better perspective on regulation of gonadal development.
3. Phospholipoprotein measurement with ALP assay in this study resulted in high individual variation, potentially due to non-specificity and low sensitivity of the assay. Enzyme-linked immunosorbent assay (ELISA) of vitellogenin which is a more sensitive and specific assay than the inexpensive ALP assay should be considered as a method of choice for vitellogenin measurement.
4. It would also be interesting to document the very early embryonic development to better document the cleavage. The movement of embryonic cells could be certainly monitored by using specific techniques targeting genes such as *Hox* gene that is a group of associated genes that determine body plan regions of an embryo head-tail axis and specify appendages (Couso, 2009).
5. Documenting post-hatching growth and development could better help to understand the annual reproductive cycle in *E. nani*.
6. Lastly and importantly, documenting the development in the closely related species and various environments is necessary to better associate changes in developmental patterns and reproductive strategies together with phylogeny and ecology in an evo-eco-devo perspective.

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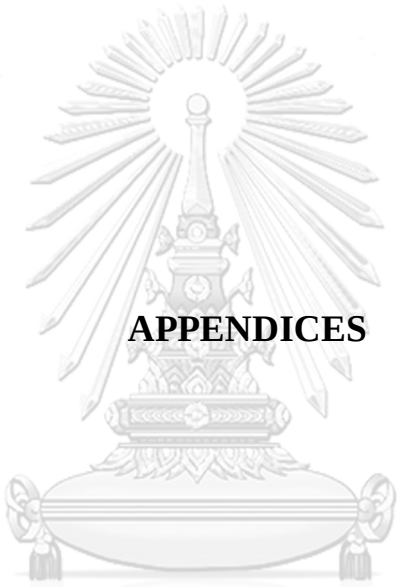
APPENDICES



Appendix A

Note on Histology of Accessory Organs at Mandibular Muscle of the Male *Esanthelphusa nani*

Mandibular organs (MOs)

In crabs, the endocrine control of spermatogenesis and testicular growth has not been yet studied *in extenso*. The mandibular organ (MO) is one of the endocrine gland that is known to respond to environment and that may have a role in the control of reproduction (Nagaraju and Borst, 2008). Methyl farnesoate is a product of MO that is known to stimulate testicular growth (Nagaraju et al., 2004; Reddy et al., 2004). However, there are still few studies done on the MO histology, and the related histological change of this organ with testicular growth is limited (Nagaraju and Borst, 2008). In this work, it was aimed to reveal histology of MO in *E. nani* and determine whether histology of MO can display change due to seasonal variation. Thus, the accessory glands on mandibular muscle that is potential to be MO has been selected to study for MO of *E. nani*.

Dissection of accessory organs

Accessory organs (AOs) of *E. nani* locate at anterior mandibular tendon base and posterior mandible in similar location of MOs to other crabs (Yudin et al., 1980; Gopal and Devi, 2018). To locate it, the reproductive tract and hepatopancreas were first removed. Since AOs were attached to the mandibular tendon by several connective tissues, MOs were gently pulled out of the tendon. The AOs were fixed in Davidson's solution overnight. Seventy percent ethanol solution was used to preserve the tissue prior to preparation for histological examination under the microscope.

Microscopic examination of accessory organs

AO was processed using the paraffin embedding method described in Appendix B: tissues were dehydrated in alcohol series, then cleared in xylene, and

embedded. The embedded AO was subjected to section at 6 μM thickness. The AO section was stained by hematoxylin and eosin (Appendix B). The Periodic acid–Schiff staining method (PAS: Appendix B) was also used since it can reveal the carbohydrate component in AO cells. AO tissues were examined and studied under light microscope and photographed by Canon 7DII camera fixed on the microscope objective.

Histology of accessory organ based on testicular stages (Fig A1)

The tissue of accessory organs (AOs) was observed microscopically, and differences were screened to see whether they could be associated with testicular stage: resting stage, developing stage, and active stage. (See Chapter IV). There are two major cell types in the AO. The cells in type I form the tubular glands, whereas cells in type II pack inside the tube structure and plays important role on AO secretion since they release their secretion.

Accessory organs at resting testicular stage (Fig. A1 A, B, and C)

AO shows the initial stage of tubular gland structure with glandular part that is less developed. The sinus cavities are present. The nucleus of cells in type I are round with peripheral distributed chromatin. The type I cell forms narrow tubes. Some of them form the tube around the mega cell type II with a large size, that can be 6 to7 times of type I cell. Cells in type II have huge and round nucleus. The large cytoplasm of type II cell is not revealed with the PAS stain.

Accessory organs at developing testicular stage (Fig. A1 D, E, and F)

The AO shows a tubular gland structure with glands that are slightly narrower to those at active testicular stage, but the tubules are longer. The sinus cavities occupy

greater portions between tubular structures. Nucleus of cells in type I is round with peripheral scattered chromatin. The cytoplasm is stained with eosinophilic color. The nucleus of type II cell is observable at this stage. Large cytoplasm of type II cell shows only pinky stain of PAS.

Accessory organs at active testicular stage (Fig.A1 G, H, and I)

The morphology of AO at this stage can be identified as mature. The tubular glands are tightly packed in the AO, and small sinus cavities are present. The nucleus of type I cell presents intensive chromatin. The type II cells are at mature stage and their nucleus are difficult to observe. Large cytoplasm of type II cell is not stained by eosin, but the PAS show pinky color on those that indicating of carbohydrate secretion.

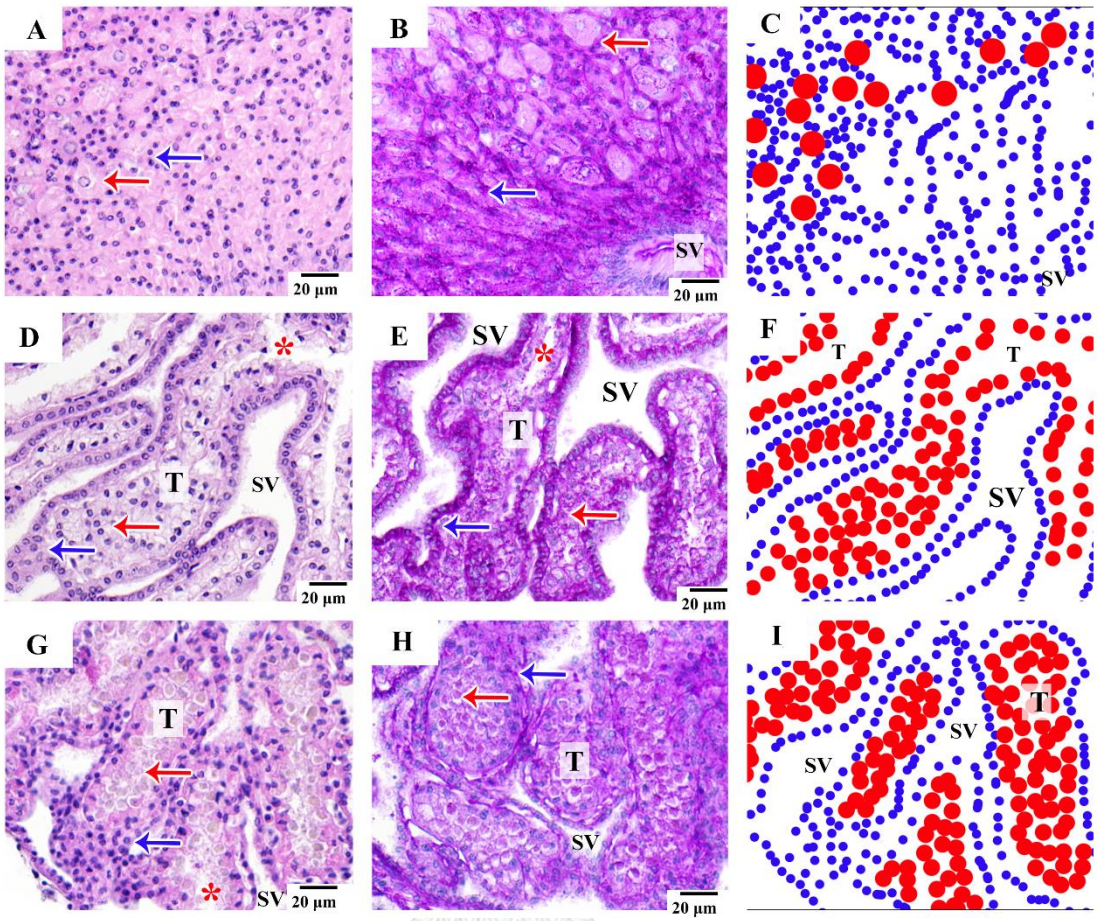


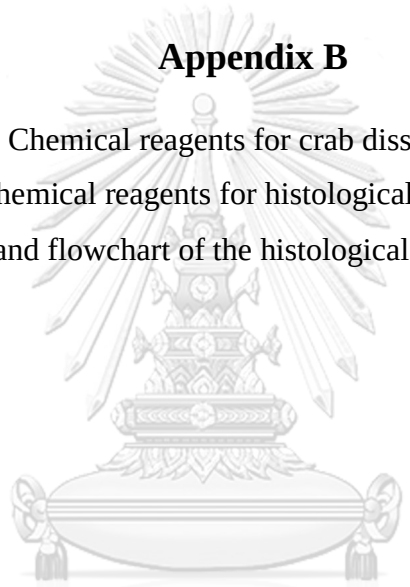
Figure A1 Photomicrograph of tubular gland structure of accessory organs. A, B, and C) The accessory organs during resting testicular stage; D, E, and F) The accessory organs during developing testicular stage; G, H, and I) The accessory organs during active testicular stage. The left side present H&E staining results. The center present PAS staining results. The right side present drawing of accessory organ structure; T: Tubular area; SV: sinus cavity; blue arrow or dot: type I cell; red arrow or dot: type II cell; (*): released area of type II cell.

Discussion and note on the accessory

Dense and less dense cells in MO cells has been identified in *Fenneropenaeus indicus* (Penaeid shrimps) (Vijayan and Diwan, 1994). Based on cell and nuclei size, two different cell types has been recognized in *Travancoriana schirnerae* (Gecarcinucidae, primary freshwater crab) (Gopal and Devi, 2018). The MO tissue of *T. schirnerae* has showed the gradual growth of tissue structure during gonad development. The tissue structure of MO present cord like arrangement without tubular formation. In present study, the accessory organs present the tubular structure. This structure is not similar to the general structure of MO that present in *T. schirnerae*. In conclusion, the AO found in the study cannot be identified as the MO in *E. nani.* Other accessory organs nearby mandible area of *E. nani* should be further investigated.

Appendix B

Chemical reagents for crab dissection,
chemical reagents for histological process,
and flowchart of the histological process



I: Crustacean saline

a.	NaCl	12	grams
b.	KCl	0.4	gram
c.	$CaCl_2$	0.4	gram
d.	$MgCl_2 \cdot 6H_2O$	0.5	gram
e.	Distilled water	1	L

***Check the pH; if necessary, add $NaHCO_3$ (about 0.17 g) to bring the solution to pH 7.4

II: 250 mM Citric acid for anti-hemolymph clotting

a.	Citric acid	0.525	grams
b.	Distilled water	10	mL

III. Davidson's solution

a.	Formaldehyde (37-40%)	500	mL
b.	Glycerol	100	mL
c.	Glacial acetic acid	100	mL
d.	Absolute ethanol	300	mL

IV. Hematoxylin and eosin reagents

1. Eosin Y, 1 % Solution:

a.	Eosin Y dye	1	gram
b.	Distilled water	100	mL

2. Delafield Hematoxylin Solution:

a.	Hematoxylin	4	grams
b.	Absolute ethanol	25	mL
c.	Ammonium alum	6	grams
d.	Distilled water	400	mL
e.	Absolute methanol	100	grams
f.	Glycerin	100	mL

3. Differentiator

a.	70% ethanol	100	mL
b.	Conc. hydrochloric acid	4	drops

V: Periodic acid and Schiff's reagent

1. Periodic Acid Solution:
    a. Periodic acid 1 gram
    b. Distilled water 100 mL
2. Schiff's Reagent:
    a. Basic fuchsin 1 gram
    b. Sodium metabisulphite 1.9 grams
    c. 0.15M HCl 100 mL

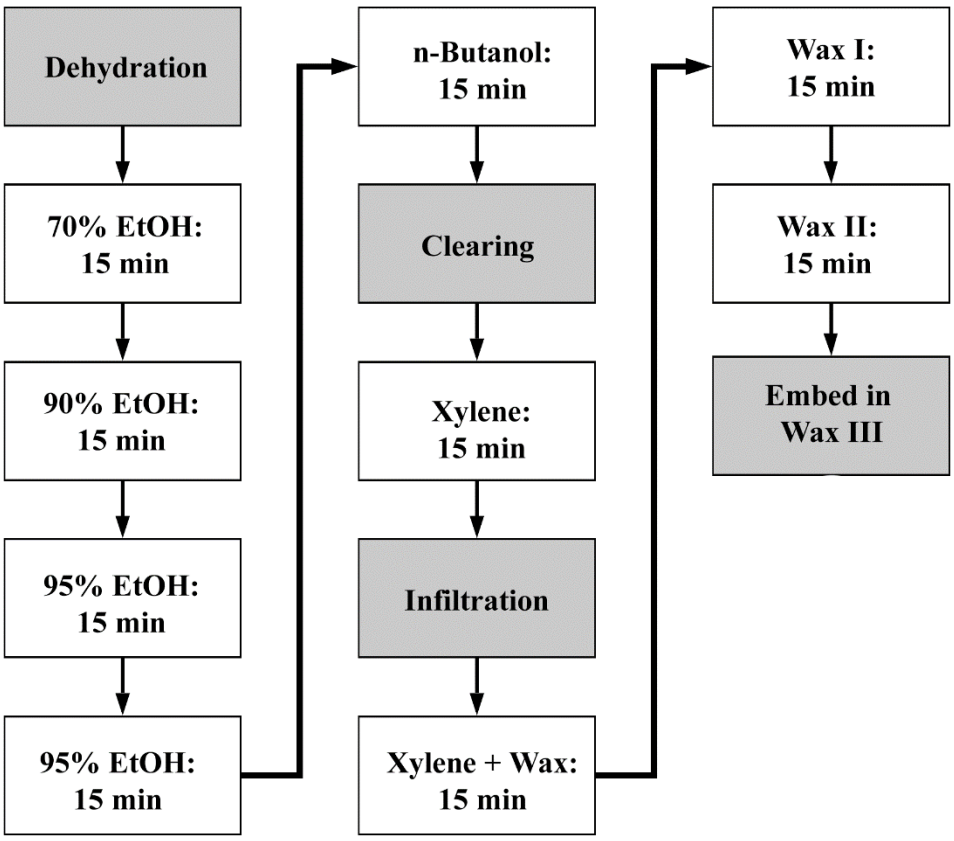


Figure B1 A flowchart of paraffin embedding for *E. nani* testis, ovary and accessory organ

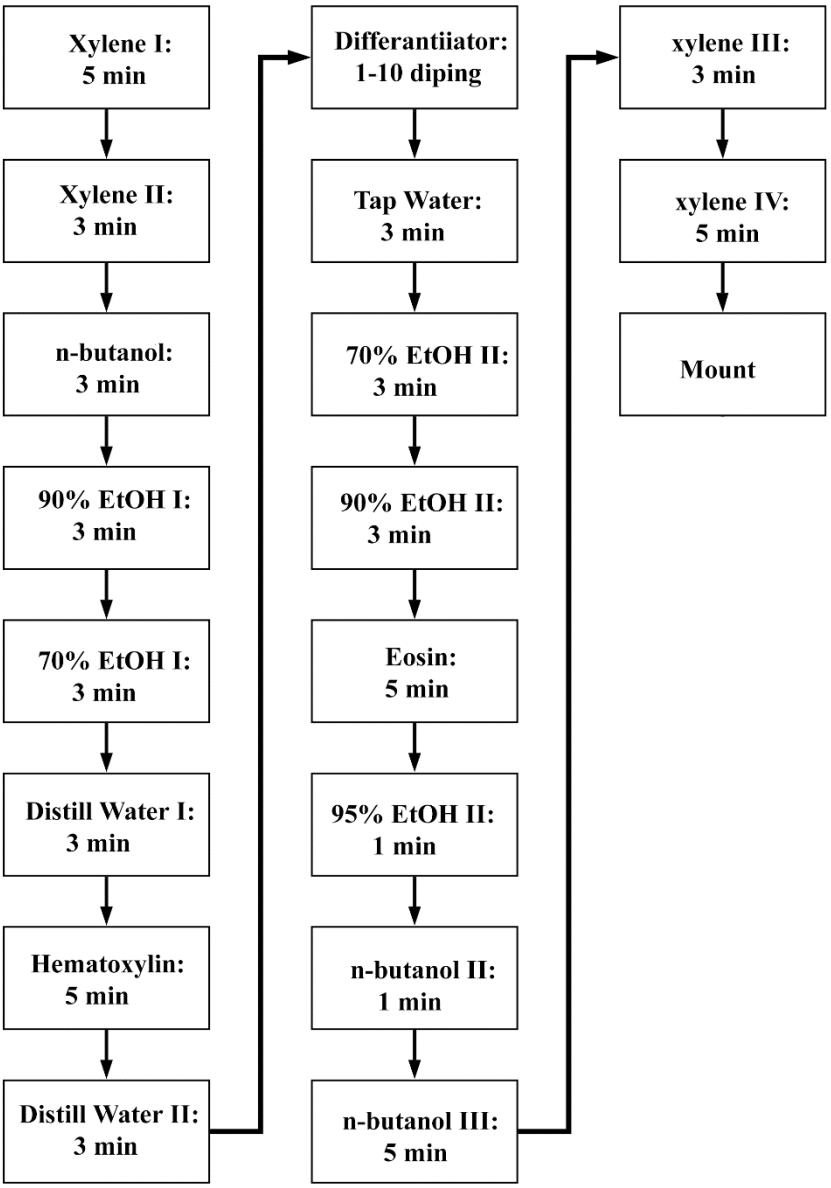


Figure B2 A flowchart of hematoxylin and eosin staining for *E. nani* testis, ovary and accessory organ

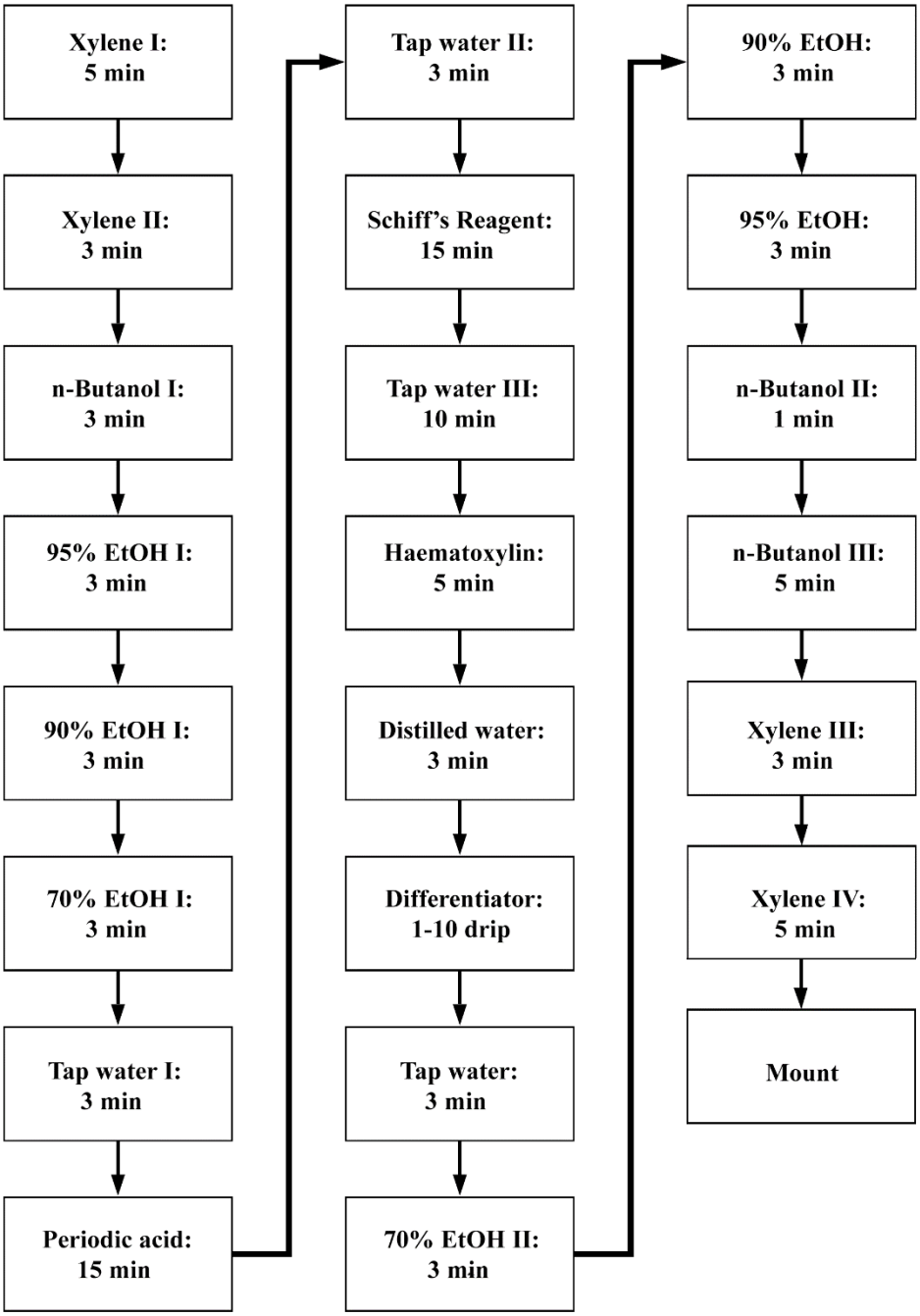
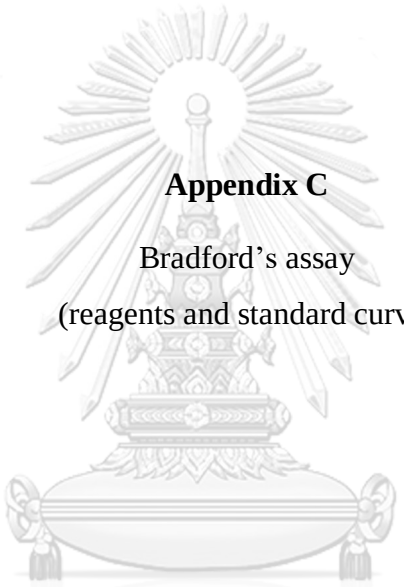


Figure B3 A flowchart of periodic acid and Schiff's reagent staining for *E. nani* testis and accessory organ



Appendix C

Bradford’s assay

(reagents and standard curve)

I: Buffer solution for total protein measurement (phosphate buffered saline (PBS), pH 7.2)

a.	NaCl	8.0	grams
b.	KCl	0.2	gram
c.	Na_2HPO_4	1.44	grams
d.	KH_2PO_4	0.24	gram
e.	Distilled water (final volume)	1	L

***Check the pH; if necessary, add HCl/NaOH to bring the solution to pH 7.2

II: Stock albumin standard 25 µg/mL

a.	Bovine serum albumin conc. 2.0 mg/mL (Thermo®#Lot# PA196779)	312.6	µL
b.	Distilled water (to make 25 mL in a volumetric flask)		

III: Stock for quality control sample 1 (7.5 µg/mL)

a.	Stock albumin standard 25 µg/mL	300	µL
b.	Distilled water (to make 10 mL in a volumetric flask)		

IV: Stock for quality control sample 2 (2.5 µg/mL)

a.	Stock albumin standard 25 µg/mL	100	µL
b.	Distilled water (to make 10 mL in a volumetric flask)		

V: Specific document of Bradford's reagent (Sigma®)

a.	Bradford reagent Lot# SLBL4590V (no mixing required)		
b.	Amount of protein sensitivity	1-400	µg/mL
c.	Time of incubation	5 min.	
d.	Optimum reading absorbent wavelength	595 nm.	

Precision of Bradford's assay

Coefficients of variation (CVs) of assay

Intra-assay variation: 1.35%-2.00% (<10%,)

Inter-assay variation: 2.40%-3.20% (<15%,)

Standard curve

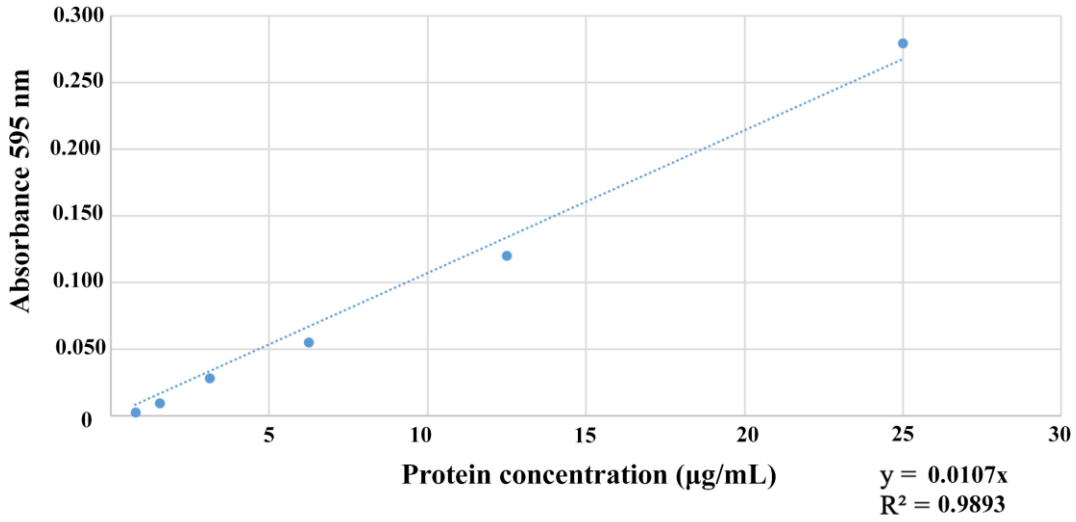
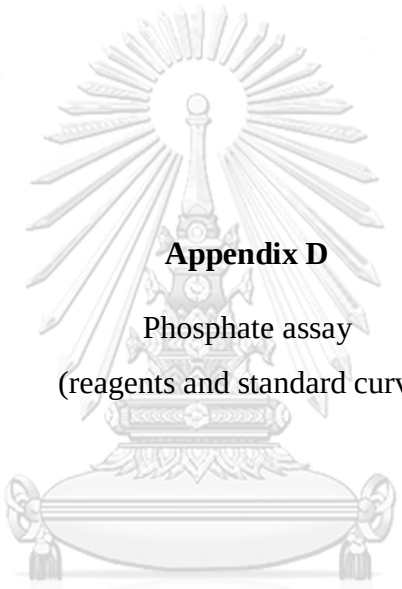


Figure C Representative standard calibration curve of Bradford's assay



Appendix D

Phosphate assay

(reagents and standard curve)

I: Components of phosphate assay kit (Catalog Number MAK308A)

c. Malachite green reagent (Sigma®#Lot# 308BI02A22)

d. 1 mM phosphate standard (Sigma®#Lot# 308BI02A22)

II: Premix solution of phosphate standard

c.	1 mM phosphate standard	40	µL
d.	Distilled water	960	µL
	(final volume in 1 mL)		

III: Standard phosphate preparation in assay

Standard #	Premix standard vol. (µL)	Water vol. (µL)	Final vol. (µL)	Phosphate conc. (µM)
1	200	0	200	40
2	160	40	200	32
3	120	80	200	24
4	80	120	200	16
5	60	140	200	12
6	40	160	200	8
7	20	180	200	4
8	0	200	200	0

IV: Stock for quality contral sample (20 µM)

a.	1 mM phosphate standard	40	µL
b.	Distilled water	1,960	µL
	(final volume in 2 mL)		

V: Specific document of phosphate assay (Sigma®)

e.	Amount of phosphate sensitivity	0.4–50 µM phosphate
f.	Optimum reading absorbent wavelength	600-620 nm

*** Exogenous free phosphate would interfere with the assay, it is important to ensure the protein preparation, the reaction buffer, and labware employed in the assay do not contain free phosphate.

Precision of phosphate assay

Coefficients of variation (CVs) of assay

Intra-assay variation: 2.4%-3.2% (<10%,)

Inter-assay variation: 4.3%-5.2% (<15%,)

Standard curve

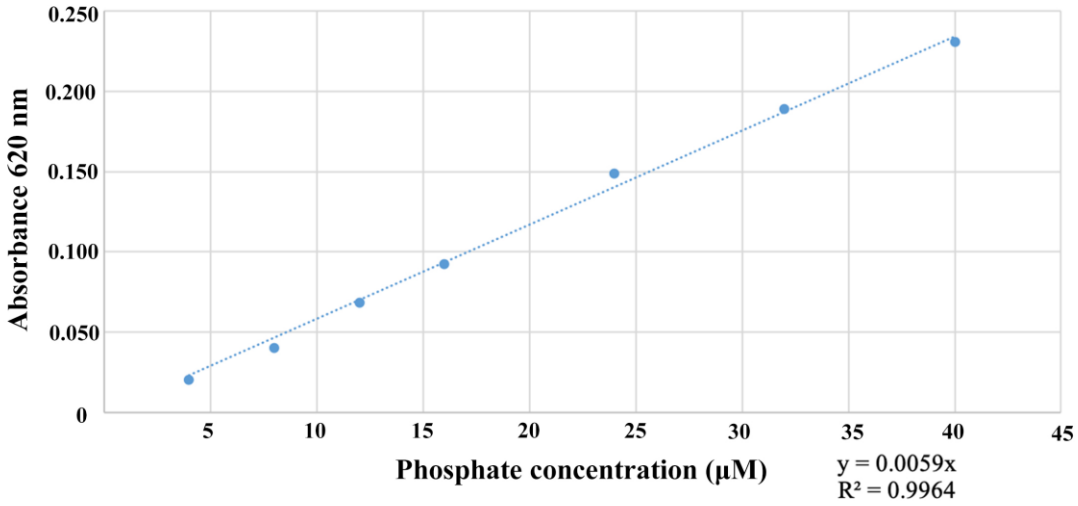


Figure D Representative standard calibration curve of phosphate assay

VITA

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PUBLICATION	JOURNAL ARTICLES:

Maneein, R., Khonsue, W., Varanusupakul, P. and Kitana, N. 2011. Association between atrazine utilization and biologic response of rice field crab Esanthelphusa nani in paddy fields of Nan Province, Thailand. Research Journal of Chemistry and Environment 15 (2): 1018-1023. [JCR Impact Factor 2011 = 0.379, M.Sc. research]

Maneein, R., Martinand-Mari, C., Claude, J., Kitana, J. and Kitana, N. Embryonic development of the continental crab Esanthelphusa nani (Naiyanetr, 1984). (submitted to Journal of Crustacean Biology, SJR Q2)

ABSTRACTS:
Maneein, R., Kitana, J., Claude, J. and Kitana, N. 2016. Larval development stages of rice field crab Esanthelphusa nani in Nan Province. The 3rd National Symposium on Biodiversity Management, June 15-16, 2016, Nan, Thailand, pp. 244-245.
(Poster presentation)

Maneein, R., Kitana, J., Claude, J. and Kitana, N. 2017. Confocal microscopic observation for stages of larval development of the rice field crab Esanthelphusa nani from Thailand. The Crustacean Society Mid-Year Meeting 2017, June 19-22,
2017, Barcelona, Spain, p. 59. (Oral presentation)

Maneein, R., Martinand-Mari, C., Claude, J., Kitana, J. and Kitana, N. 2017. Studying development of freshwater crab, Esanthelphusa nani, for understanding reproductive radiation of crabs. The 22nd Biological Sciences Graduate

Congress, December 19-21, 2017, Singapore. P. 68 (Oral presentation)

Maneein, R., Kitana, J., Claude, J. and Kitana, N. 2019. Macroscopic and microscopic analysis of ovarian cycle of the rice field crab Esanthelphusa nani (Naiyanetr, 1984). The Crustacean Society Mid-Year Meeting 2019, May 26-30, 2019, Hong Kong, p. 24. (Oral presentation)

Maneein, R., Martinand-Mari, C., Claude, J., Kitana, J. and Kitana, N. 2019. Larval morphology of the freshwater crab, Esanthelphusa nani, as revealed by confocal laser scanning microscopy. The 19th Congress of The International Federation of Associations of Anatomists. August 9-11, 2019, London, The United Kingdom (Poster presentation)

.

AWARD RECEIVED

2013: Chula Mongkut, Faculty of Science, Chulalongkorn University

2013-2017: 100th Anniversary Chulalongkorn University for Doctoral Scholarship

2016: Research award, The 3rd National Symposium on Biodiversity Management, Nan, Thailand

2016: Franco-Thai Cooperation Programme in Higher Education and Research SIAM

2016: Overseas Research Experience Scholarship for Graduate Student from the Graduate School, Chulalongkorn University

2017-2019: Graduate Student Research Scholarship under CU Academic Network in the Region

2018: CU Honorary Award for Graduated Student of Faculty of Science, Chulalongkorn University