Histology Study of Asian Clam (*Corbicula fluminea)* Reproductive Gonad

By

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A report in fulfilment of the requirements for the degree of Bachelor of Applied Science (Animal Husbandry Science) with Honours

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DECLARATION

I hereby declare that the work embodied in this Report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

_____

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I certify that the Report of this final year project entitled “Histology Study of Asian Clam (*Corbicula fluminea*) Reproductive Organ” by Nurshahiza binti Abd Kadir, matric number F14A0284 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Husbandry Science) with Honours,

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Histology Study of Asian Clam (*Corbicula fluminea*) Reproductive Gonad

ABSTRACT

Corbicula fluminea is freshwater clam that can endure with various climate and environment atmosphere. For morphology, the species is yellowish brown at outer shell and the inside is white, while the umbone area is light orange and there is purple flash along the teeth. An experiment was conducted in order to investigate oogenesis, spermatogenesis and the sexual cycle of the bivalve *Corbicula fuminea*. 40 samples were collected from Kampung Tok Uh, Tumpat, Kelantan in September 2017. Corbiculidea clam, *Corbicula fluminea* were assessed by histological analysis of gonad. Other than that, the stages of development of sex gonad were investigated throughout light microscope. Based on result obtained, 19 (47.5%) of *Corbicula fluminea* specimens were males, 15 (37.5%) females and 6 (15%) were hermaphrodites with some stages of reproductive cycle found.

KEYWORDS: Histological analysis, *Corbicula fluminea,* reproductive system, gonad, hermaphrodites

Kajian Histologi Sistem Pembiakan Gonad Kepah Asia (*Corbicula fluminea*)

ABSTRAK

Corbicula fluminea merupakan kepah air tawar yang boleh hidup di kawasan payau dan boleh bertahan dalam pelbagai iklim dan persekitaran. Dari segi morfologi, bahagian luar cengkerang berwarna coklat kekuningan dan di bahagian dalam berwarna putih, bahagian belakang berwarna oren cerah dan warna ungu sepanjang bahagian gigi. Eksperimen telah dijalankan untuk menkaji oogenesis, spermatogenesis dan kitaran seksual kerang – kerangan *Corbicula fluminea*. Sejumlah 40 sampel telah dikutip di Kampung Tok Uh, Tumpat, Kelantan pada bulan September 2017. Pembiakan kerang Corbiculidea, *Corbicula fluminea* dinilai menggunakan kaedah histologi dan tahap perkembangan jantina gonad dikaji menggunakan mikroskope. Berdasarkan keputusan yang diperolehi, 19 (47.5%) sampel *Corbicula fluminea* adalah jantan, 15 (37.5%) betina dan 6(15%) hermafrodit dengan beberapa tahap kitaran pembiakan telah dijumpai.

Kata Kunci**:** Analisis histologi, *Corbicula fluminea,* sistem pembiakan, gonad, hermafrodit

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LIST ABBREVIATIONS

GCI	Gonad condition index
CI	Condition index
COI	Cytochrome c oxidase 1
ppt	part per thousand
mg/L	Milligram per litre
mm	Millimetre
µm	Micrometer
g	Gram
cm	Centimetre
cm^2	Centimetre square
h	Hour
m	Metre
min	Minute
ml	Millitre

LIST OF SYMBOLS

°C	Celcius
%	percentage

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Members of *Corbicula fluminea* species employ a variation of reproductive approaches (Keeler, 2014). *C.fluminea* is a freshwater clam but has been known to happen in brackish water. Genus Corbicula is less demanding compare to native mussels on environmental irregularity factors with their sensitivity to low oxygen atmosphere. For reproduce, the *Corbicula* necessitate temperature more 16°C and decrease metabolism with respiration more than 30°C.

For shell morphology of *C.fluminea*, the outer shell is yellowish brown and the inside is white, while the umbone area is light orange and there is purple flash along the teeth. Ishibashi et al. (2014) had presented the yellow type is corresponding with the type *Corbicula* above. Representing another type *C.fluminea*, it external surface is yellowish brown and the inside is purple. Taxonomic status of *C. fluminea* is presented in Table 1.1.

Table 1.1: Taxonomic status of *Corbicula fluminea* (Burkhead, 2015)

Scientific classification	
Kingdom	Animalia
Subkingdom	Bilateria
Phylum	Mollusca
Class	Bivalvia
Order	Veneroida
Family	Corbiculidae
Genus	*Corbicula*
Species	*Corbicula fluminea*

Coloured bivalve with beautify of the shell by different, concentric sulcations, anterior and posterior lateral teeth with many fine serrations with small light physical are about characteristics of shell morphology that describe by Burkhead, (2015).In South – western, United State, dark shell morphs was found but in limited amount. AnDong et al. (2001) stated that yellow and brown shell colour alters among samples that taken from Sichuan Province in China. Outside of the yellow morphs, it were straw yellow and the inside is white while for brown morphs, the dark brown placed in outside and purple inside. Yellow and brown morphs reported as triploid and tetraploid after all analyses done.

Due to this high oxygen demand, Asiatic clams typically inhabit well oxygenated streams and lake shoals. The maximum acceptable salinity for *C.fluminea* is 13 ppt, but this concentration may only be endure for a short time (Keeler, 2014). Favourable habitat must uphold temperature within range of 2 to 30 °C, calcium concentration of at least 6 mg/L and pH greater than 5.6.

Adult *Corbicula* are hermaphrodites (both male and female) which are able of both cross and self – fertilization, thus it required only a single individual to start a population. Fertilization happens in the paleal cavity with larvae brooder in the inner dermibranchs. Larvae are released into the water column and afterwards anchor themselves to deposition, vegetation or rigid layers via a mucilaginous byssal thread structure (Cataldo & Boltovskoy, 1998). Juveniles are relatively small, around 250 µm, completely formed with fully grown of outer case of bivalve until 6 to 10 mm length.

The timing of commonly observed bivoltine reproductive periods varies regionally, but the first event commonly happens in late spring to early summer and then repeated in late summer to early autumn. The adult Asian clams can live 1-5 years and spawn 1-3 times per breeding season. An individual adult can produce 1000 to 100,000 juveniles per years. These characteristics also authorize Asian clam to obtain high densities in unpredictable marine habitat and impressionable to environmental fluctuations. They flourish in well oxygenated river and oligotrophic lakes with sandy or gravel substrates. Another characteristics that make Asian clam an efficient invader is resistant to dehydration and can survive extends periods in moist sediments thus eliminating water drawdowns as a management method.

However, a widespread various of parasites in the Corbiculidae and their accomplishment as disease transmitter is due to distributive and increase amount of *Corbicula* sp. (Sousa et al,. 2008). In endemic areas, it classified as public health problem as it cause severe disease in man because of some parasites forms from *Corbicula* species. According to Carney et al. (1980), the disease attack the body because of eating clams raw or barely cooked.

From CAB International, *C.fluminea* is known as competitive with another species of bivalves for food and space and the *C.fluminea* can change benthic substrata. It is also high survivality compare to native species of bivalves in tolerate contaminate environments. Previous studies shown the filter feeding of *C.fluminea* had important removal of suspended particles from water column but, this species had function in increase rate of sedimentation and to continue the water flow, it need more frequent dredging which give a serious effect on the river ecosystem and increase the costs.

Because *C. fluminea* is an active clam which can spread very fast and harmfully which can endure with various climates and environment atmosphere, attain high community densities, the purpose of the experiment was to establish represent reproductive organ of *C. fluminea* and to establish it histological gonad.

1.2 PROBLEM STATEMENT

Corbicula fluminea is a freshwater bivalve mollusc and it's widely recognized as 'etok' at Malaysia. It also one of traditional food that consume by people in Kelantan. However, *C.fluminea* now difficult to find because of insufficient of seed. So, this research will help in improve production of seed.

1.3 HYPOTHESIS

H_0 = There is mature *C.fluminea* present on population.

H_1 = There is no mature *C.fluminea* present on population.

1.4 OBJECTIVE

To identify reproductive organ of *C. fluminea.*

1.5 SCOPE OF STUDY

To determine gender dominated in population to help in improve production of broodstock.

1.6 SIGNIFICANCE OF STUDY

Identification of reproductive organ will provide the database of gravid Asian clam for reproduction strategy in seeds production because production of sloping downwards because overexploitation by human.

1.7 LIMITATION OF STUDY

The recent study was aimed to identify reproductive organ of *C.fluminea*. However, there a limitation in finding 'etok' because of not suitable period to collect *C.fluminea*.

CHAPTER 2

LITERATURE REVIEW

2.1 TYPES OF CLAM

Freshwater clam in the family Corbiculidae are among the most numerous macro invertebrates in many of the world's river, lake and estuarine habitats. Due to their extraordinary high densities, these clams can be important in benthic production, nutrient cycling and water sanitization (Byrne et al., 2000). *Corbicula australis* is significance part of the microbiota of the river system in the Southeastern region.

Jones et al. (1994) and Sousa et al. (2009) describe that the *C. fluminea* is acting as an ecosystem architect which as an aggressive species bivalve that can spread very quickly that has colonized comprehensive marine ecosystem with tremendous ecological and commercial effect. According to Crespo et al. (2017), *C. fluminea* as type of freshwater clams, which is competent to conquer top debouchment with distinct environment of salinity and temperature. Reproductive plasticity is an extreme demonstrated by *C. fluminea*, which familiar to most invasive species. For unstable habit likes Santa Cataline stream, the clam rapidly respond to proper environment atmosphere for gametes spawning. Cao et al. (2017) said that crisis in establishing pattern of gamete delivery as well as gonad return to normal is a clearly evidence for unstable habits above.

The Hard clam, *Meretrix lyrat* possessions to Veneridae family. This clams is found at certain areas in Sarawak, Malaysia such as Buntal Village and Kabong. *M.lyrate* live in seaside and debouchment areas. Histological method is most an accurate process to decide sex and gametogenic evolution. This method is compulsory to dye the sample of tissue before gametogenic level can be resolved. Biological action in mollusc can be impact and regulate by habitat environment which is one of important factor (Hookham et al., 2014; Rashid et al., 2009). Additionally, previous studies was planned to describe reasons of natural condition such as salinity, temperature and food as an sign that effect gametogenic evolution of species (Saxby, 2002; Thiet & Kumar, 2008).

Corbicula leana from Moe Prefecture, Japan is genus *Corbicula* produce non-reductional spermatozoa. Hermaphroditic and broods its larvae in the inner demibranches is the special mode of reproduction for *C.leana* compare to another species. Accordding to Okamoto and Arimoto, (1986) possibility that *C.leana* reproduces by gynogenesis, which would accountable for the odd chromosome number. Sakai et al. (1994) describe *C.leana* external surface is dark brown and the inside of the shell is deep purple. Analysis of chromosome numbers suggest that *C.leana* (3n=54 in somatic cells and 18 in meiotic cells) from Mie Prefacture are triploids and diploids. Compare of analysis of allozyme variation between *C.leana* with *C.japonica* and *C.sandai,* the result show that the *C.leana* has much lower genetic variability than these two.

Genus *Corbicula* were separate into two species which *C. fluminalis* and *C. fluminea*, each with a different reproductive system. *Corbicula* are native of freshwater and estuarine habitats in south Asia Africa, the Indian subcontinent. *Corbicula* species are successful immigrants in new areas (McMahon, 2000). *C.fluminalis* and *C.fluminea* distinguish based on conchological characters, contrasting reproductive strategies and ecological differences. Morton (1982) reported *C.fluminea* to be essentially an inhabitants of streams, but also adapted to life in almost all types of freshwater habitats. *C.fluminalis* is largely an inhabitant of lentic large estuaries, showing a higher tolerance to salinity (Rajagopal et al., 2000). These two species do not live together in any ecosystem. The hermaphroditic *Corbicula* clams have a very unique made of reproduction androgenesis (Ishibashi et al., 2003).

Corbicula japonica is one of the best – known bivalve mollusc in the Far East of Russia. Based on Dzyuba et al. *(*2013), Japanese corbicula is well acclimated and rather resistant to changes in salinity and temperature. In habits estuaries, lakes and lagoons that connect to the sea and is a widespread euryhaline species. In addition, the corbicules have valuable pharmacological properties which attract the attention of scientists as biologically active substances in seafood. For the rational use of the Japanese corbicula in the areas and its potential for breeding in the Kievka estuary, one needs known the biology of this mollusc, first of all, those of its reproduction with which this work deals.

2.2 REPRODUCTION

In the study of O'Foighil (2000), molecular data show that *C.australis* is clearly nested within aclade of *Corbicula* together with other freshwater lineages including *C.fluminea, C.sandai* and *C.leana.* In this investigation, *C. australis* was collected from two population in upper Nepean River, Douglas Park (34° 11' 715" S; 150° 42' 757" E) and Menangle (34° 07' 357" S; 150° 44' 521" E). Seasonal gonad were collected from both sites in March, May, August and October. The reproduction condition of individual sample was assessed by investigate of the visceral mass and the gills were observed for the presence of embryos. This investigation to determine the brooding cycle of *C. australis* to provide baseline data to assess the reproductive response of the clams to planned experimental releases from upstream impoundments.

The investigation was observed in Santa Catalina Stream, Argentina (36°53'64.5''S - 59°55'25.22''W). Specimens were taken from April 2003 – April 2005 excluding November 2004. Modesto et al. (2013) estimated that environments that showed high temperature values integrate with low salinity and low water flow can encourage higher densities of *C. fluminea.* 60% to 100% of observed samples was hermaphrodites (with oogenic, spermatogenic and mixed follicles), which most permanent individuals were females. The appearance of males was only recorded on October 2004 and January 2005. Hermaphroditism is usually more often in freshwater than marine bivalves. Reproductive strategies of *Corbicula* species was different based on the type of environment they inhibit. McMahon (1982) reported the *Corbicula* can be hermaphrodite with larval incubation inside gill chambers in freshwater but in estuaries, they are usually dioecious, oviparous and non – breeding. Other abilities factors for established variation in the reproductive cycle of *C. fluminea* could be

causes of water temperature (Baba et al., 1999; Dzyuba et al., 2013), plentiful of phytoplankton (Cataldo & Boltovskoy, 1998; Mouthon & Parghentanian, 2004) or even variations of metallothionein concentrations between individuals.

Afzalina and Diomira through communication said that numerous short – period studies reproduction had been conducted at Asajaya Laut and Buntal with the usage of gonadal condition index (GCI). Razor clams, *Solen regularis* was collected from both site, Asajaya Laut (N 01° 36' E 110° 36') and Buntal (N 01° 36' E 110° 22'). A maximum number of 30 specimens were collected at the two week interval or monthly. Live specimens of razor clams were transported back to laboratory for GCI study. On the observation of mean GCI pattern, it is suggested that razor clams at both locations have five stages of reproductive cycle. For sexes razor clams cannot be distinguished externally. Through microscopic observation, the gonad appeared in two different colourations where female was whitish with milky texture, while male was beige with granular texture. The reproductive cycle of razor clams is an annual cycle. They spawned at least three times during the spawning period from end of March – April to September.

For experiment of Hard clam, *Meretrix lyrata,* approximately 30 matured of clams with shell length 36.0 mm to 76.0 mm were gathered at the Buntal Village, Kuching, Sarawak, Malaysia site (N' 01° 42' 18.6" E 110° 22' 03.6") every month from May 2013 to April 2014. Specimens of *M.lyrata* quickly transported to the laboratory and were cleaned from unpleasant sediment and organism. Based on investigation, reproductive biology of *M.lyrata* from Sarawak in present study was overcome by male. Matured peak for *M.lyrata* mostly at the early of the year and spawning exist at the middle of year to September.

(J.-K. Park & Ò. Foighil, 2000) describe that the Asian clam, *C. fluminea* is one of the world's most well-known and extensive invasive organism. (Ishibashi et al., 2003) also describe that Asian clam species is most broadly spread over and highly tend to spread quickly and known also from North America, South America Europe and Australia. This experiment to study the reasons that reassurance the reproductive efforts. The clams collected at Lake Tahoe (39.13° N, 120.05° E). *C. fluminea* species were sampling at four sites which Lakeside, Maria Bay and Nevada Beach. To calculate eggs and developed fertilized larval forms, we dissected the gills approximately 40 clams per site across sampling dates. Clams were dissected occasionally when the target size class not fully satisfied. The specimens were collected using an Ekman grab sampler of capture area 468 cm^2 to accomplish aim this experiment to present new data on allocation of *C. fluminea*. For each site, samples were collated in triplicate from 5 locations throughout lateral transects of the river. The specimens were collected at evenly space intervals, starting and finishing estimate 1 cm from each bank of the river.

A latest study of the chromosome of *C. leana* revealed that it has 54 somatic chromosomes but in gonads, three homologous chromosomes from 18 trivalents. The synapsis of three homologous chromosomes should be complete so judging from its karyotype, *C. leana* is triploid species. *C. leana* were taken in Japan from an irrigation continuation with no specific species which maternal chromosomes exhaustive and spermatozoa started evolution of the eggs but the paternal genome does not embroiled of the offspring. *C. leana* can happen by parthenogenesis without self – fertilization by eggs and sperm. In the identical hermaphroditic follicle or the genophore, there may abundant spermatozoa take part in the start-up of the mature eggs and egg cleavage because reproduction of this species was classified as parthenogenetic and is only

stimulus for parthenogenesis. Hermaphroditic *C. leana* clams are ovoviviparous, but can also reproduce by releasing oocytes and spermatozoa from the siphon, these then undergo external fertilization (Houki et al., 2011).

Complex series of *C. fluminalis* were obtained from the river Rhine at Lobith (between April 1991 and May 1992) and from the river Waal al Nijemen (between September 1991 and November 1992) and for *C. fluminea* from the river Lek at Lekkerkerk between January 1992 and January 1993. For *C. fluminalis,* circumstances of the gonads was categories into five main stages, following the fact established by Morton (1982) which primordia, developing, mature, spawning and hermaphrodite. It is dioecious, with a small portion of hermaphrodites. *C. fluminea* is a concurrently hermaphrodite, which incubate fertilised eggs inside its inner demibranchs and deliver pediveliger. There four different levels were defined which immature, mature, larvae and spawning. *C. fluminalis* and *C. fluminea* showed distinct spawning periods which *C .fluminea* delivere its pediveliger larvae from May to September, while *C. fluminalis* delivered its gametes during October – December and March – April. Otherwise to *C. fluminea, C. fluminalis* body mass gain from December – March, when chlorophyll – a concentrations were very low, reveal that alternative food sources was important for this species other than algae. *C. flunimea,* which shows brood care, assign more energy resources to reproduction before spawning than *C. fluminalis.*

The material for the *Corbicula japonica* study was sampled in estuary of the Kievka River in May 2010 – April 2011 at the depth of 0.6 m (Dzyuba et al., 2013). The previously identified stages of reproductive cycle in bivalves we used: sexual inactivity, early gametogenesis, active gametogenesis, the pre - spawning stage and the spawning stage. In the males cross sections of the acini and gonad tube areas

occupied by spermatogonia, spermatocytes, spermatids and mature sperm cells were distinguished and the ration of the these area were evaluated. With the development of the reproductive process the gonads are purple in females and yellowish – cream colour in males. Important oogenesis in Japanese corbicula was the participation of auxiliary cells of the gonad in the ensuring the trophoplasmic growth of oocytes. Regularly, in *C. japonica* spotted individual sperm with 2 flagella. It is known that in other *Cobicula* species, example *C. fluminea,* two flagella per sperm is the norm (Dzyuba et al., 2013). The individual sperm cells with two or more flagella probably developed due to their polyploidy with two or four nuclei, which may result from incomplete meiotic division or other pathogenic.

2.3 HISTOLOGY

The following procedure used by most researcher for experiment to investigate the reproductive gonad using histological analysis. At least 40 clams collected and transferred to the laboratory and put in 0.45 µm filtered sea water for 24 h in order to clean out their stomach. Each data of individual clam were recorded which total wet weight (g), shell length from anterior to posterior (cm) and width from dorsal side to ventral side (cm) and for each clam was numbered to avoid mistake when the data recorded.

For processing, the soft tissue of each clams were removed from the shell using a scalpel and fixed in Bouin's fluid for 24 h. After rinse in distilled water, the body of the soft tissue were cut into two to provided transverse section of the visceral mass, which consists of the gonad, renal gland and digestive tract and section of the gill and mantle. Then following the method of Howard & Smith (1983), the tissue dry out with a various of ethanol solutions, dealcoholized in butanol and embedded in paraffin wax. Wax block sliced into 5 to 7µm section and stained with Harris's haematoxylin (Humason, 1979) and eosin. The gonadal slide were examined with light microscope (10 – 40x objectives) to determine sexes.

Another study by (Grner et al., 1999) cell calculation methods were similar to those of Jones *et al* (1986) and Haggerty *et al,* (1995) can be used. For processing, using method described above from Humason, (1979), Howard & Smith, (1983). Then, for identification sex, use cell quantification method, which for spermatogenesis, if the cell touched the X axis of an eyepiece reticule that was moved across the approximate centre of 10 acini per male specimen, they were estimated and identified by using

optical microscope and 1000x magnification. Based on size, shape, intensity of staining and location in the acinus, the cell were identified as spermatocytes and multinucleated inclusion. Qualification of oogenesis formation and maturation of ova measured by diameter of oocytes along a transect across the entire section of gonad. The plane of section passed through the nucleus that contain oocytes only were calculated and for measurement used a ruled eyepiece reticule. Transect were run along the Y axis of the reticule cross hairs. A transect was defined as the width equal to 10 units of measure on each side of the Y axis.

CHAPTER 3

MATERIALS AND METHODS

3.1 LOCATION OF *Corbicula fluminea*

Asian clam, *Corbicula fluminea* were collected from Kampung Tok Uh, Tumpat, Kelantan (6° 07' 59" N 102° 09' 9" E). Sample were collected from one site only. The slide preparation was conducted in Laboratory Histopathology at Faculty of Veterinary Medicine and observation for slide conducted at Biology Laboratory, Universiti Malaysia Jeli Campus, Kelantan.

3.2 SAMPLING OF *Corbicula fluminea*

Asian clams were initially found using a vertical tow net that scooped sediment into the net (Minchin, 2014). About 30 to 40 mature of *C.fluminea* were collected from the mid – tide area toward low – tide area of the study site. Length (mm) and weight (g) of each clam had been recorded.

3.3 MATERIALS

3.3.1 EQUIPMENT

List of equipment that were used in this experiment are net, electronic weighing balance, centrifuge tubes, scalpel, light microscope, model Leica from United State of America and formalin.

3.3.2 MACHINES

List of machine that were used in this experiment are auto stainer, model Leica from United State of America (US), manual rotary microtome, model Leica from US., paraffin embedding section, model Leica from US, cold plate, model Leica Eg 1130 from US, tissue floatation water bath, model Leica from US, and automatic tissue processor, model Leica from US.

3.4 METHODS OF HISTOPATHOLOGY

3.4.1 FIXATION

Slide preparation begins with fixation of tissue specimen. This is important step that to make sure cell and tissue components maintained their structure and therefore slaves down tissues degradation during cross linking and coagulation. The tissues

from clam were transferred into centrifuge tube containing 10 ml formalin with 90 ml distilled water with ratio 1:9. The tissues adequately fixed at least 24 hours.

3.4.2 DEHYDRATION

After fixation, the tissues were dissect using scalpel to fit into tissue cassette, then the tissues were immersed in series of graded alcohols which 50% alcohols, then 70%, 80%, 95% absolute alcohol and then transferred to xylene in increasing concentrations of alcohol to remove the water and formalin from the tissues by using automatic tissue processor from Leica. These steps take about two to three hours each.

3.4.3 EMBEDDING

The tissue block were embedded with a media that function as a support during the sectioning process. Paraffin wax were using for this purpose. By using processing machine paraffin embedding section from Leica, once the paraffin wax was infiltrated the tissue, the position was carefully located and surrounded with extra wax using mold to form a block. Then, tissues were attached to a tissue cassette. For each cassette has the patient biopsy code. The paraffin wax were solidified the tissue blocks at room temperature or to facilitate the clamping of the tissue to its base, the tissue blocks placed on refrigerated surface.

3.4.4 SECTIONING

This step to get the thin section by using manual rotary microtome from Leica. The surface of the paraffin cube was cut by a knife that placed in microtome and produce a series of thin section of very accurate thickness. The objective is to produce a continuous ribbon of sections stick to one another by their leading and trailing edges.

The tissue block was trimmed first to remove excess paraffin from embedding tissue cassettes. Then, the microtome pre – set at 5 µm to slice tissue sections. The slice tissue sections form a continuous ribbon and these are picked up and transferred in warm water bath. The tissue were float on the surface of water and then a glass slide placed underneath the tissue. Slides were clearly labelled and dried overnight to make sure the tissue adhere to the slide.

3.4.5 STAINING

Staining used to determine cell shape, size and arrangement of microorganisms. The several slide can be placed for one staining dish. For one staining dish, it take about 48 minutes to complete. Haematoxylin and eosin (H & E) was used to examine the sections of tissue by using auto stainer program from Leica. The slide were placed on 20 slide rack and put in auto stainer for staining process. Haematoxylin stains cell nuclei blue, while eosin stains cytoplasm, connective tissue and other extracellular substances pink or red.

There were several step for staining process that need to be complete in order to get the result. For step 1 until 3, which contain xylene 1 and 2 with 100% ethanol, it

for cleaning and takes 11 minutes. Step 4 until 7 which contain 100%, 90%, 80% and 70% of ethanol, it was immersed in 1 minutes for each percent ethanol to water enter the tissue. Then, the slide washed for 3 minutes. Next, the slide rack containing the slide were immersed in Harris haematoxylin to detect nucleus in the slide. The slide were washed for second time for 3 minutes. Slides immersed in MX – AQ for 10 seconds to clean the microorganisms except nucleus. The nucleus were detected when the slide had a purple colour. The slide washed for the third time and then immersed in blue buffer for 1 minutes to concentrate the purple colour. The slide washed for fourth time. Next, to detect cytoplasm, the slide were immersed in eosin for 3 minutes. Slides then immersed in 80%, 90% and 100% of ethanol for dehydration process. Last step for staining process is the slide were immersed again in xylene 1 and 2 to clean it.

3.4.6 MOUNTING

After the staining complete and tissue dehydrate, the slide were covered with a cover slip. This step called as mounting and it the last step in preparing the slide. This step necessary to prepare permanent specimen. This step involving placing a thin cover slip over stained sections and dried.

3.5 MICROSCOPIC EXAMINATION OF *Corbicula fluminea*

The slide that contain tissue section were observed under the light microscope model Leica from US by using 10x, 20x and 40x magnification in order to find the gonad.

CHAPTER 4

RESULT AND DISCUSSION

4.1 DETERMINATION OF GENDER DISTRIBUTION

For *C. fluminea*, 40 specimens were examined which had 19 males, 15 females and 6 hermaphrodites. The collected individuals ranged from 1.5 – 2.9 mm.

Table 4.1. Standard deviation, mean, length and weight of male *Corbicula fluminea.*

Length (mm)	Weight (g)
2.08	2.57
2.05	2.7
2.1	2.14
2.1	2.48
2.07	2.4
2.04	2.83
1.9	1.81
1.72	2.52
2.1	2.47
2.05	2.44
1.9	1.84
2.88	1.91
1.88	1.92
2.39	2.18
2.27	3.43
1.94	4.35
2.1	2.53
2.12	2.75
2.39	2.28
mean ± stdev = 2.11 ± 0.249	mean ± stdev = 2.50 ± 0.596

Table 4.2. Standard deviation, mean, length and weight of female *Corbicula fluminea.*

Length (mm)	Weight (g)
2.48	4.38
1.95	1.47
1.71	2.57
2.11	2.29
1.97	2.49
2.05	2.85
2.75	2.63
2.17	2.05
2.16	2.83
2.20	2.80
2.11	2.60
2.04	2.48
2.23	3.07
2.49	2.54
1.72	1.47
mean ± stdev = 2.14 ± 0.276	mean ± stdev = 2.57 ± 0.681

Table 4.2. Standard deviation, mean, length and weight of hermaphrodites *Corbicula fluminea.*

Length (mm)	Weight (g)
2.29	3.34
2.10	2.87
1.73	1.78
1.80	1.75
2.52	4.39
1.90	1.92
mean ± stdev = 2.62 ± 0.306	mean ± stdev = 2.68 ± 1.063

Based on table 4.1, the percentage of males (only spermatogenic follicles) was 47.5% (19) of examined in all samples, while for females (only oogenic follicles) 37.5% (15) and remaining individuals were hermaphrodites (with oogenic and spermatogenic follicles) only 15%. Regardless of the evidence that *C. fluminea* may achive very large quantity under local environment, environmental elements may have big influence on its species densities and disposition. McMahon (2002) reveal that contradictory to what can be assumed for offensive species, *C. fluminea* has comparatively low physiological compromise to change in sterile factors, such as temperature, salinity, exposure towards air, pH, and calcium and dissolved oxygen concentration. A fact stated can be a reason for resulting in the higher amount of male compare to female and hermaphroditic.

4.2 OBSERVATION OF GONAD

In histology sections of the hermaphroditic gonads, the area of testicular tissue was smaller than of the ovarian tissue as shown in Figure 4.1, by see the label a and c.

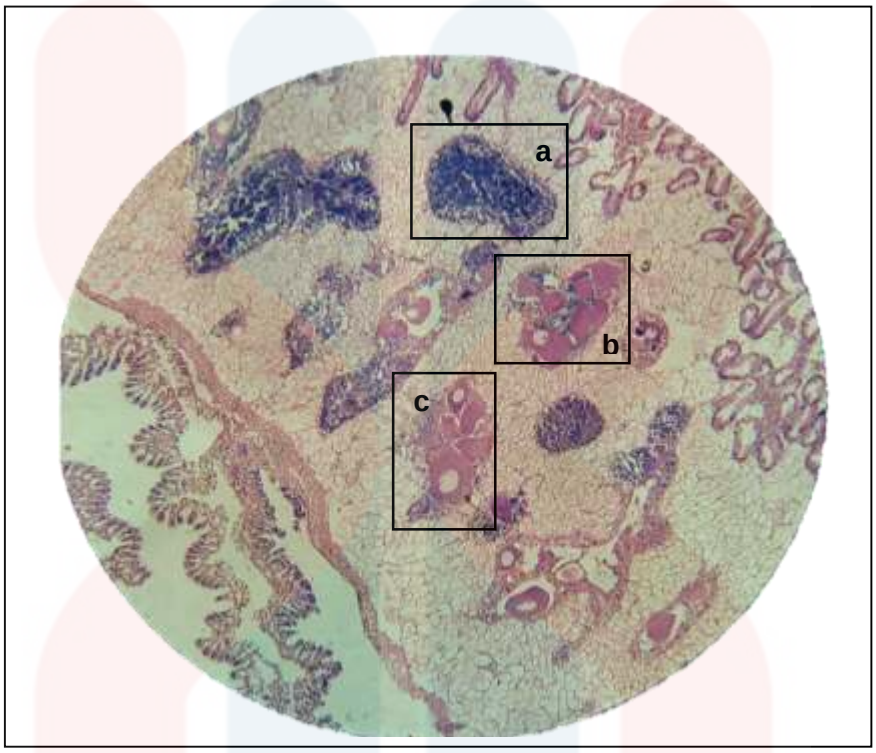

Figure 4.1. Hermaphroditism in *Corbicula fluminea* with 40x magnification. General aspect of the visceral mass showing (a) spermatogenic follicle, (b) mixed follicle and (c) oogenic follicle.

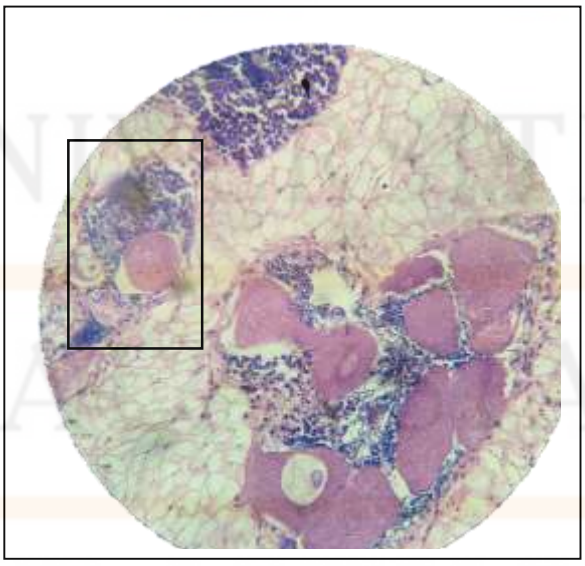

Figure 4.2. Image of an embryo during egg cleavage, surround by number of sperms in the hermaphroditic follicle with 40x magnification.

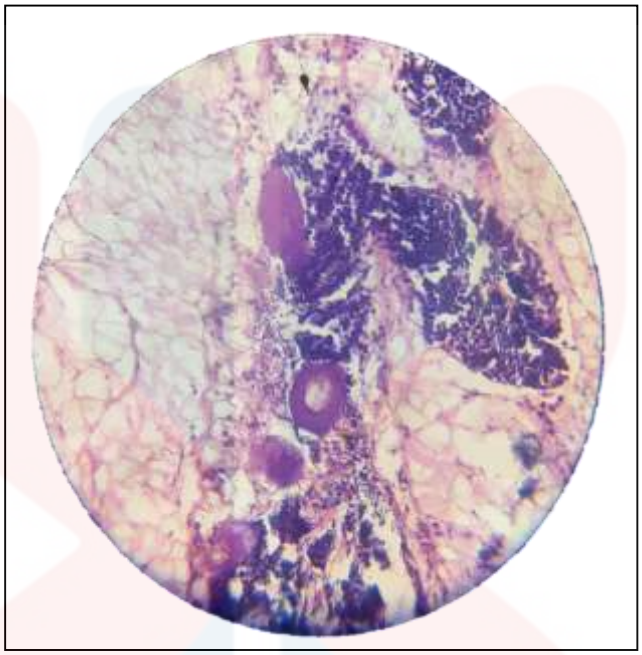

Figure 4.3. Image of hermaphroditic follicles with spermatocytes and spermatid in the lumen and interfollicular connective tissues with 40x magnification.

The males in hermaphroditic clam species cannot mate with females, as in ordinary bisexsual species. If there are no chances for fertilization, these males will soon become extinct. In this reproductive mode, outcrossing does not involve the mixture of nuclear genomes from two parents (Komaru et al., 2006). Thus, replacement of the nuclear genome can occur if males release spermatozoa and fertilize oocytes from hermaphrodites. In the gonad hermaphroditic clams, the ovarian tissue predominated. The spermatogenesis activity in hermaphrodites was lower than that in males. Figure 4.2 show incompletely spawned follicle, appear of an embryo uncleavaging which encircle by an amount of sperm. A spermatocytes develop into spermatids. At this time, spermatid located in the middle of lumina of hermaphroditic follicles were fulfilled with a little oocytes, the interfollicular connective tissue were also widely fulfilled as shown in Figure 4.3. Sex ration may alter because of environmental factors or exposure to chemical substances during early stages of development. For

example, low temperature can induce male biased sex ratios, as shown experimentally in hermaphrodite fish (Robert & Harrington, 1967)

The spermatogenic regions were usually located in the terminal portion of the ascini along the edge of the visceral mass with a few scattered sperm in other regions. Spermatogonia, which happen at the follicle walls are oval cell with nucleus full of chromatin granules. The nucleus boundaries were contain of the nucleolus. Spherical cells with a big homogenous nucleus which are called as spermatocytes. Spermatids are polyhedral with a homogenous nucleus (Park & Chung, 2004).

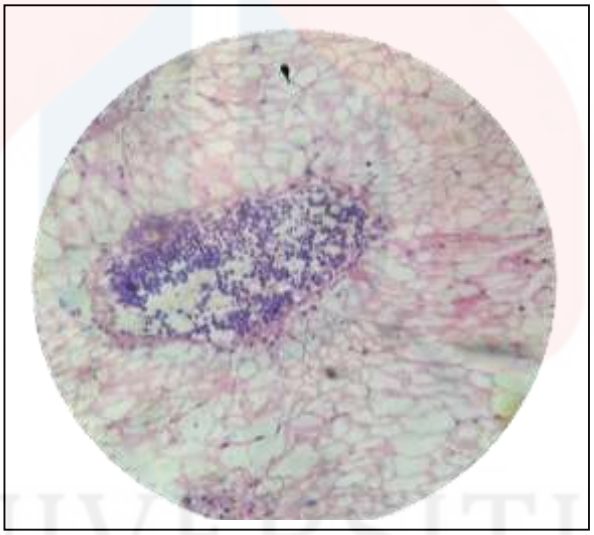

Figure 4.4. Image of spermatozoa in the follicular wall with undifferentiated mesenchymal cells and eosinophic cells in follicle with 40x magnification.

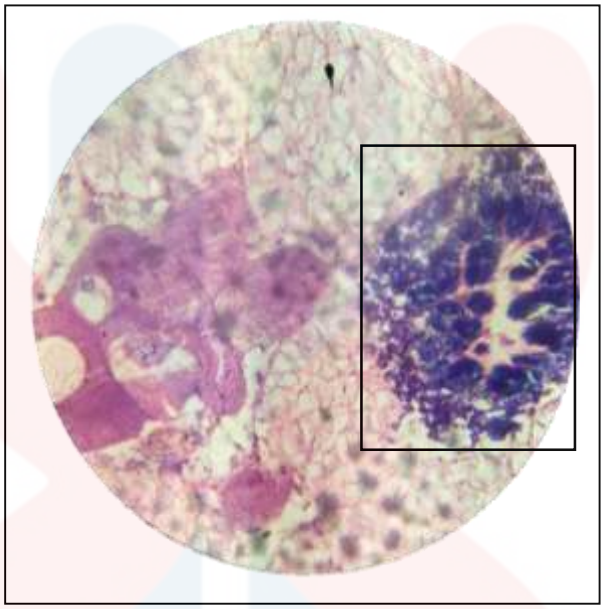

Figure 4.5. Image of testis in the mature stage, showing the needle shaped sperm clusters in the spermatogenic follicles with 40x magnification.

The big oval nucleus was consist inside spermatogonia which takes place in wall of the spermatogenic or hermaphroditic follicles. Undifferentiated mesenchymal cells and eosinophilic cells were existed closed by the spermatogonis and spermatocytes as shown in Figure 4.4. The shape of the nucleus changed slowly and begin to be a little elongated and narrow in the beginning differentiation stage of spermatid. Figure 4.5 show process after spermatogenesis, batches of needle - shaped sperm was formed inside the male follicles by a number of spermatozoa. Male reproductive tissue was less significant than female tissue. At this time, the gonoduct was takes place closed by the spermatogenic follicle and the follicular ganglia, correlated with neurosecretion and the distinction of complex inneryated nerve structure current spermatogenesis (Park & Chung, 2004).

Oogenesis happen inside oogenis and hermaphroditic follicles. An oogenic take place closed the outer muscular layer began to start the evolution forward the visceral mass. Oogenic are spherical and with distribute chromatin and they are very indistinguishable to spermatogonia.

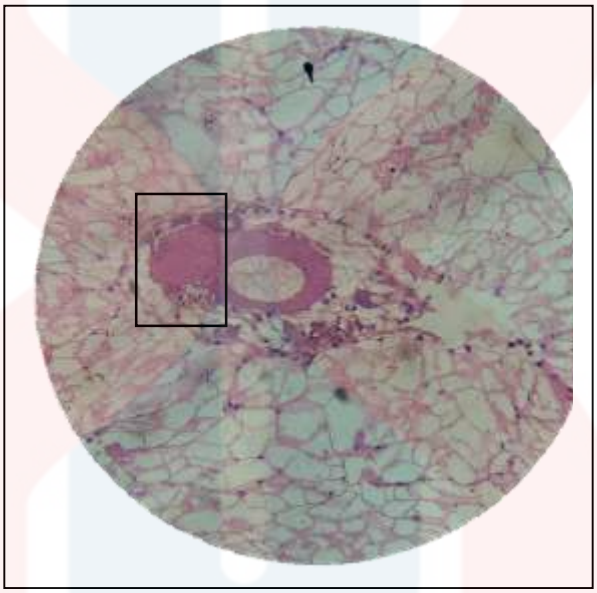

Figure 4.6. Image of oognia in the follicular walls showing undifferentiated mesenchymal tissues and eosinophilic cells with 40x magnification.

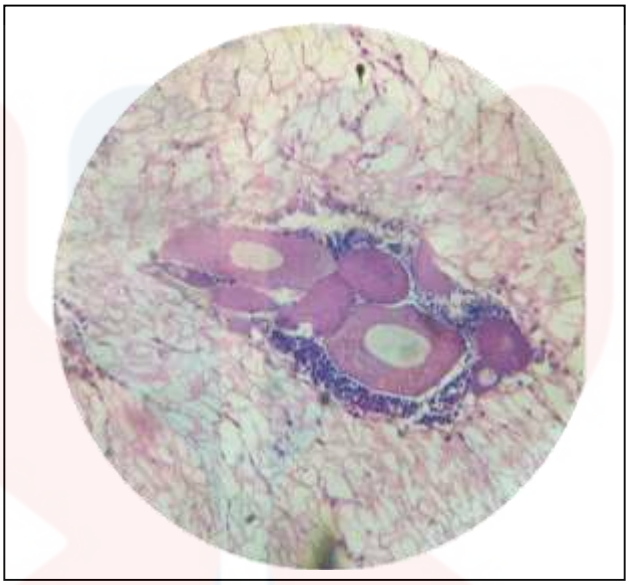

Figure 4.7. Image of early vitellogenic oocytes with oogenic follicles and hermaphroditic follicle with 40x magnification.

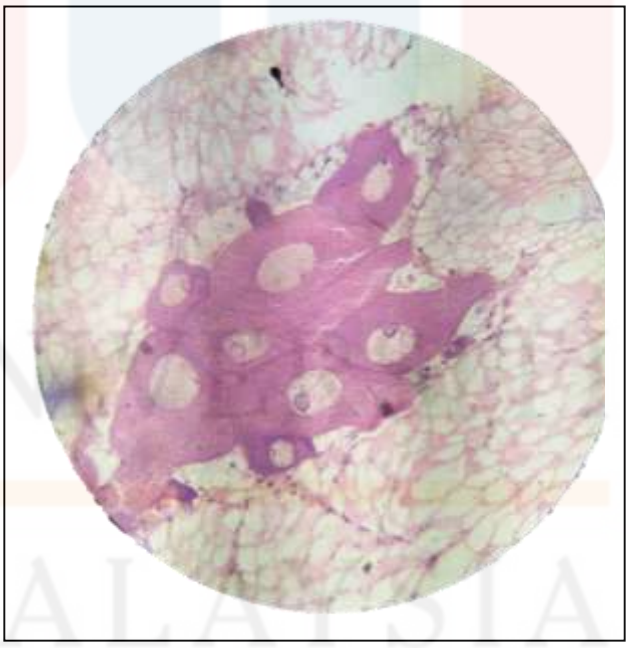

Figure 4.8. Image of a mature oocyte, with a large nucleolus in the nucleus and numerous yolk granules in the cytoplasm with 40x magnification.

In the centre of round nucleus, there was contain a nucleolus with appear number of oognia along the follicular walls. In the nucleus, the appearance of one nucleolus was different, although the cytoplasm of the oogonium was very poor technique during staining. Currently, an amount of undifferentiated mesenchymal tissues and eosinophilic cells were both located closed the follicle walls as shown in Figure 4.6. The oognium developed into the previtellogenic oocyte. A round nucleus consist of one or more small eosinophilic nucleoli through the nuclear envelope appear inside previtellogenic oocytes and the cytoplasm started to develop in volume. Undifferentiated mesenchymal and eosinophilic granular cells were plentiful on the follicular wall. There were extensively spread into interfollicular connective tissues closed the follicles. Inside oogenic or hermaphroditic follicles, as shown in Figure 4.7, it shows the early vitellogenic oocytes grew and become eosinophilic oval or pentagonal. Figure 4.8 explain about mature oocytes had one big nucleolus, 3 – 4 small nucleoli in the nucleus with the viteline envelope of the ripeness oocytes was encircle with a gelatinous substances (Park & Chung, 2004).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

As a conclude, reproductive biology of *Corbicula fluminea* from Kampung Tok Uh, Tumpat, Kelantan had successful achieved and dominated by male as data indicate in Table 4.1. Most gonad development had reached at mature stage in September which *C. fluminea* collected and prepared for histopathology. The clams dominated by male because of males succeed in reproducing or in other words, the male gonads is genetically fixed. Hermaphrodite was the lowest abundance in the samples and the possibility it occur because of environmental factors or exposure to chemical substances during early stages of development may cause the sex ratio. These results on the gametogenesis of this invasive species may be a fundamental tool for development of strategies and programs implementation to increase their proliferation in Malaysia.

5.2 RECOMMENDATION

This study, previous had been conducted in more than 2 years (Cao et al., 2017; Rajagopal et al., 2000). So, it is suggest that further research could be done with a prolong duration in order to obtain more accurate result. (Cek & Sereflisan, 2009) reported that in Asia, number of dioecious of individual was low because of environmental factors such as temperature, food, salinity, nutrition and geographical location. To proof for this statement, the further study can be investigate the reproductive organ of *Corbicula fluminea* that rear in different environment and temperature and see either the result is dominated by males, females, hermaphrodite or sexually-undifferentiated. To obtain a good result, the timing of life cycle is important and avoid collect the clam during resting period because it will empty their gonad tissue because of spawning period had complete.

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APPENDICES

Figure A.1 Dissect of sample into cassette

Figure A.2 Tissue immersed in series grades of alcohol using automatic tissue processor.

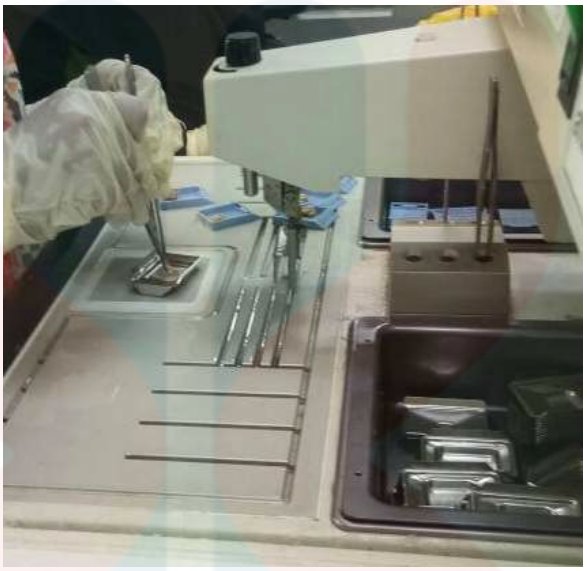

Figure A.3 Tissue embedded in mold to form a block by using paraffin wax.

Figure A.4 The paraffin wax solidified the tissue block on refrigerated surface.

Figure A.5 Trimming process to remove excess paraffin from cassettes using microtome.

Figure A.6 The cassettes placed in cold plate after trimming.

Figure A.7 Process sectioning tissue use microtome

Figure A.8 Tissue section picked up and transferred in warm water bath and glass slide place underneath the tissue.

Figure A.9 Arrange the slide in slide rack and put in auto stainer for staining process.