SPERM CRYOPRESERVATION OF TROPICAL OYSTER, *Magallana bilineata* (Röding, 1798)

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SPERM CRYOPRESERVATION OF TROPICAL OYSTER, *Magallana bilineata* (Röding, 1798)

by

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LIST OF ABBREVIATIONS

μm	Micrometer
μL	Microliter
°C	Degree Celcius
psu	Practical Salinity Unit
%	Percentage
mL	Milliliter
L	Liter
RM	Ringgit Malaysia
h	Hour
min	Minute
g	Gram
cm	Centimeter
M	Molarity
±	Plus or minus
C_1	the concentration of the stock solution
C_2	the final concentration of the diluted solution
V_1	the volume to be removed from the concentrated stock solution
V_2	the final volume of the diluted solution
DMSO	Dimethylsulfoxide

PENGAWETAN KRIO TERHADAP SPERMA TIRAM TROPIKA,

***Magallana bilineata* (Röding, 1798)**

ABSTRAK

Musim monsun yang tidak menentu boleh menggangu pengeluaran tiram secara komersial di Malaysia melalui pengubahan masa pengeluaran sperma and telur tiram. Untuk memelihara industrian pengeluaran tiram di Malaysia bagi pengeluaran yang berterusan, bank penyimpanan benih tiram boleh menjadi satu penyelesaian dalam masa terdekat. Pengawetan krio dilakukan terhadap sperma tiram tropika, *Magallana bilineata* (Röding, 1798). Sperma *M. bilineata* dicairkan dalam larutan Ringer marin yang diubahsuai dan ditambah air laut bertapis 25 psu 1 µm pada nisbah 1:3. Kemudian, 200 µL campuran sperma *M. bilineata* ditambah dengan dua agen pengawetan krio (CPA) tembus (Gliserol dan Dimetilsulfoksida (DMSO)) dan tidak tembus (Glukosa dan Sukrosa) dalam empat kepekatan (5%, 10%, 15%, 20%) pada nisbah 1:1, 1:3 dan 1:5 untuk mengkaji kesannya dalam dua kaedah pembekuan; a. 10 minit dalam mandian Etanol- ais kering sahaja dan; b. 10 minit dalam mandian Etanol-ais kering dan kemudiannya dimasukkan ke dalam nitrogen cair. Eksperimen kawalan yang ditetapkan adalah campuran 200 µL sperma segar yang ditambah air laut bertapis 25 psu 1 µm pada nisbah 1:1, 1:3, 1:5. Selepas pembekuan, sperma dicairkan dalam mandian air pada 40°C dan kemudian disenyawakan dengan telur tiram *M. bilineata* segar. Penilaian daya saing telah dilakukan melalui pewarnaan Eosin-Nigrosin pada sperma dan diperhatikan di bawah pembesaran 1000x pada mikroskop cahaya. Peratusan daya saing sperma tertinggi selepas pencairan didapati dalam eksperimen kawalan (68.72 ± 8.47%). Eksperimen nisbah 1: 3 sperma kepada CPA menghasilkan peratusan daya saing sperma tertinggi di antara eksperimen nisbah sperma kepada

CPA yang lain. Peratusan daya saing tertinggi mengikut jenis CPA adalah 35.76 ± 5.04% (DMSO 5%), 31.98 ± 7.75% (Gliserol 10%), 16.23 ± 6.65% (Glukosa 5%) dan 5.01 ± 2.93% (Sukrosa 10%) di mana semuanya adalah lebih rendah secara signifikan daripada kawalan (P <0.05). Peratusan persenyawaan tertinggi didapati dalam eksperimen pembekuan dengan mandian Etanol-ais kering di mana Gliserol menunjukkan peratusan persenyawaan tertinggi dalam semua nisbah sperma: CPA. Peratusan persenyawaan tertinggi dicapai dalam Gliserol 10% dengan 33.84 ± 13.59% (Eksperimen sperma kepada CPA pada nisbah 1: 1), Gliserol 10% dengan 21.49 ± 7.48% (Eksperimen sperma kepada CPA pada nisbah 1: 3) dan Gliserol 15% dengan 20.08 ± 16.98% (Eksperimen sperma kepada CPA pada nisbah 1: 5). Peratusan persenyawaan tertinggi dalam eksperimen nitrogen cair didapati dalam Gliserol 10% dengan 12.04 ± 5.63% (Eksperimen sperma kepada CPA pada nisbah 1: 3) dan berbeza secara signifikan (P <0.05) dengan peratus persenyawaan dalam eksperimen mandian Etanol-ais kering. Kemampuan persenyawaan sperma *M. bilineata* selepas pencairan adalah secara umumnya rendah dan didapati lebih rendah dalam rawatan CPA selain Gliserol. Walaubagaimanapun, kajian ini telah membuktikan bahawa pengawetan krio boleh dilaksanaka pada sperm tiram *M. bilineata*. Kajian ini akan dapat menambahkan pengetahuan mengenai pengawetan krio terhadap tiram tropika dan boleh digunakan sebagai kajian asas bagi pengoptimuman selanjutnya untuk menubuhkan satu bank penyimpanan benih tiram dalam masa terdekat.

SPERM CRYOPRESERVATION OF TROPICAL OYSTER,

***Magallana bilineata* (Röding, 1798)**

ABSTRACT

Unpredictable monsoon seasons can disrupt the production of commercially farmed oyster in Malaysia by altering the synchronization of gamete production. To better preserve the oyster farming industry in Malaysia for continuous production, an oyster storage seed bank could be a viable solution in the near future. Cryopreservation was carried out on the sperm of tropical oyster *Magallana bilineata* (Röding, 1798). *M. bilineata* sperm was diluted with a modified Ringer's solution to 25 psu 1 μm filtered seawater ratio of 1:3. 200 µL *M. bilineata* sperm was added with two permeable (Glycerol and Dimethylsulfoxide (DMSO)) and non-permeable (Glucose and Sucrose) cryoprotectants (CPA) in four concentrations (5%, 10%, 15%, 20%) at the sperm to CPA ratio of 1:1, 1:3 and 1:5 to study its effects in two freezing methods; a. 10 mins in Ethanol-dry ice bath only and, b. 10 mins in Ethanol-dry ice bath and immediately into Liquid nitrogen). The control experiment was 200 µL fresh sperm to 25 psu 1 μm filtered seawater used in place of CPA at the ratios of 1:1, 1:3 and 1:5. The frozen sperm was thawed at 40°C in a water bath and was then fertilized with fresh *M. bilineata* oocytes. Viability assessment was carried out via Eosin-Nigrosin staining on sperm and observed under 1000x magnification on a light microscope. The highest post-thaw sperm viability was found in the control (68.72±8.47%). The 1:3 sperm to CPA ratio experiments yielded the highest viability among the other sperm: CPA ratios. The highest viability by CPA are 35.76±5.04% (DMSO 5%), 31.98±7.75% (Glycerol 10%), 16.23±6.65% (Glucose 5%) and 5.01±2.93% (Sucrose 10%), all of which are significantly lower than the control ($P<0.05$). The highest

fertilization was found in the Ethanol-dry ice bath only where Glycerol was shown to produce the highest fertilization in all sperm: CPA ratios which are Glycerol 10% with 33.84±13.59% (Sperm to CPA ratio of 1:1 experiment), Glycerol 10% with 21.49±7.48% (Sperm to CPA ratio of 1:3 experiment) and Glycerol 15% with 20.08±16.98% (Sperm to CPA ratio of 1:5 experiment). The highest fertilization in the liquid nitrogen experiment was found in Glycerol 10% with 12.04±5.63% (1:3) and is significantly different ($P<0.05$) from the value in the Ethanol-dry ice experiment. The post thaw fertilization ability of *M. bilineata* sperm was generally low throughout the experiment and has been shown to be significantly lower in some CPA treatments. However, this study shows that cryopreservation can be adapted to *M. bilineata* sperm. The availability of this study will fill the knowledge gap on cryopreservation on tropical oysters and can be used as a baseline study for further optimization for the establishment of an oyster seed storage bank in the near future.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

In recent years, the promotion of ecological stewardship (Lucas, 2015; Risius *et al*., 2017; Bronnmann & Asche, 2017) has given rise to a demand in sustainable aquaculture practice involving bivalves (Shumway *et al*., 2003; Santeramo *et al*., 2017; Froehlich *et al*., 2017). Naturally a filter feeder, bivalves do not require processed fish meal feed. Instead, bivalves can trap available suspended food particles such as phytoplankton, micro-zooplankton, bacteria, detritus and dissolved organic matter (DOM) such as sugars and amino acid (Gosling, 2003). By doing so, bivalves could improve water quality in the water column by removing particulates and unwanted nutrients from the water column (Anderson *et al*., 2006; Dumbauld *et al*., 2009; Gomes *et al*., 2018).

Of the bivalves commercially farmed, oysters are a popular choice mainly attributed by its distinct briny taste profile (Yuasa *et al*., 2018) and high nutrition meat (Asha *et al*., 2014; Venugopal & Gopakumar, 2017). Chemical analysis showed that about 50% of the solids in oyster meat comprise of protein and less than 20% of lipids (Galtsoff, 1964). The meat also contained sodium, potassium, calcium, phosphorus, iron, iodine, magnesium, manganese and zinc (Galtsoff, 1964; Nurnadia *et al*., 2013) which are vital minerals for humans' neurodevelopment, bone health, immune function, body composition and tissue metabolic status (World Health Organization & Food and Agriculture Organization, 2004).

Oysters have ecological and economical importance which contributes to its demand to be farmed. Oyster reefs such as those in Chesapeake Bay are an important structural component of estuaries which provide ecological services such as habitat for mussels, barnacles and sea anemones, shelter for larval stages of fish and crustacean, food for animals, water filtration, shoreline stabilization and coastal defence (North *et al*,. 2010; Beck *et al*., 2011; Baggett *et al*., 2014).

Since the colonial era of United States of America (1800s), oysters were abundant and were harvested as food. Voracious harvesting of oysters at Chesapeake Bay gave rise to direct economic impacts such as employment of workers to work on oyster farms and sales of product (Murray & Hudson, 2013). Once a cheap food source for the poor which saved slaves from starvation, this remarkable piece of American heritage is now coveted by the rich worldwide (Conlin, 1980; National Research Council, 2004).

In Malaysia, the total production of edible oysters was 33.6 metric tonnes in 2016 (Department of Fisheries Malaysia, 2016). The growing demand of oysters in Asian countries like Malaysia, particularly in the hotel and catering sector has given rise to oyster product imports over 1199.48 metric tonnes, valued at RM 20,429,544 in the Year 2015 (Pawiro, 2010; Department of Fisheries Malaysia, 2015; Malaysia External Trade Statistics, 2015) from top producing countries such as the Republic of Korea, the United States of America, Chile, China and Japan (Stanton *et al*., 2010; Department of Fisheries Malaysia, 2015) on top of domestic production. There is a high potential for the oyster culture industry to succeed in Malaysia from its demands for high quality protein source (Bisant, 2010) and with the worldwide trends for more

sustainably produced food among its growing socially conscious consumers catching on (Agriculture and Agri-Food Canada, 2012).

In order to ensure continuous oyster production, spat availability plays a vital role in sustaining the industry. Spatfall prediction for wild oyster populations may be a challenge to tropical oyster farmers (Angell, 1986; Humphreys *et al*., 2014). In Malaysia, most of the oyster seeds are gathered from the wild until a point in time when Malaysia faced insufficient seed stock and needed to import from Thailand and Myanmar, which are also currently facing the same difficulties (Tan *et al*., 2014). Tropical oyster studies with regards to oyster gonadal maturation suggests that rainfall and salinity, possibly contributed by pre and post monsoon seasons could have influenced the reproductive cycle of the oysters (Ganapathi Naik & Gowda, 2013; Paixão *et al*., 2013). Unsynchronized reproduction cycles were observed between male and female tropical oysters under rainfall and salinity variations (Paixão *et al*., 2013), resulting in farmers leaving it all to chance when selecting wild broodstock for culturing activities.

Since the 1970s, efforts to cryopreserve oyster gametes began (Lannan, 1971) and have advanced tremendously since. Cryopreservation refers to suspended animation of structurally intact living cells by subjecting the samples to very low temperatures (Pegg, 2015; Jang *et al*., 2017). Bright prospects for this line of study include establishing viable commercial oyster seed storage to be used during seasonal variations that led to non-availability of natural seeds and oyster genetic improvement (Tiersch, 2008; Labbé *et al*., 2018). Most cryopreservation studies on oysters were conducted on Pacific oyster, *Magallana gigas* sperm (Gwo, 2001; Hassan *et al*., 2015). Studies on the cryopreservation of oyster oocytes, embryo and larvae albeit scarcer

compared to that of sperm, have been carried out with varying post-thaw survival success (Tervit *et al*., 2006; Horváth *et al*., 2012; Paredes *et al.*, 2013; Labbé *et al*., 2018). Commercial scale cryopreservation studies of oyster sperm with high fertilization rates (Adams *et al*., 2004; Dong *et al*., 2005) have been carried out and deemed feasible (Dong *et al*., 2007; Adams *et al*., 2009). Nevertheless, all cryopreservation efforts would be ineffective if the actual preserved samples are not able to grow out normally. Suquet *et al*. (2014) demonstrated that it is possible to grow out cryopreserved oyster larvae into adults with reproductive capabilities like those of uncryopreserved ones.

To date, only one reported cryopreservation study has been published on tropical oysters where Yankson and Moyse (1991) briefly mentioned the optimal cryoprotecting agent for tropical oyster, *Magallana bilineata*. The prospect of tropical oyster cryopreservation might have been bleak in the past but it has never been a better time with current available resources to re-evaluate the prospects of broadening tropical oyster cryopreservation especially in recent years where unpredictable weather and worse monsoons have hit our Malaysian shores.

1.2 Objectives of study

In an attempt to increase oyster production in Malaysia, the full understanding of the product's life cycle, market demand, along with all possible challenges and solutions, including hypothetical ones are needed. One of the recent realities of the oyster farming scene in Malaysia showed a nearby farm in Sungai Merbok, Kedah, Malaysia suffering tremendous setback due to mortality following salinity fluctuations in the river system it is farmed in. This farm also supplies some mature broodstock to

a commercial hatchery in Penang, Malaysia, which could in turn suffer from declining spat production, jeopardizing the oyster industry in Malaysia as a whole. Cryopreservation could be solution to establish an oyster seed storage bank for the oyster farmers, especially during the unpredictable monsoon seasons or from the impacts of climate change.

The species of oysters cultured there are *Magallana belcheri* and *M. bilineata*. *M. bilineata* was chosen for this study mainly due to its importance as a commercial species in Malaysia.

The objectives of this research are:

1) To determine viability of tropical oyster *M. bilineata* sperm undergoing cooling in ethanol-dry ice and in liquid nitrogen

2) To determine fertilization ability of cryopreserved tropical oyster *M. bilineata* sperm undergoing cooling in ethanol-dry ice and in liquid nitrogen

CHAPTER 2

LITERATURE REVIEW

2.1. Oyster culture in Malaysia

Efforts to farm oysters in Malaysia started in 1988 as a pilot project to increase fishermen income (Yatim, 1993). Today, major edible oyster producing states in Malaysia are Kelantan, Terengganu and Pulau Pinang (Figure 2.1). Kedah state is an emerging oyster producer in Malaysia. Its production could soon be rising, as a Knowledge Transfer Programme (KTP) was launched in Kedah by the Malaysian Government to enable local communities to generate secondary income (New Straits Times, 2014; Tan, 2015).

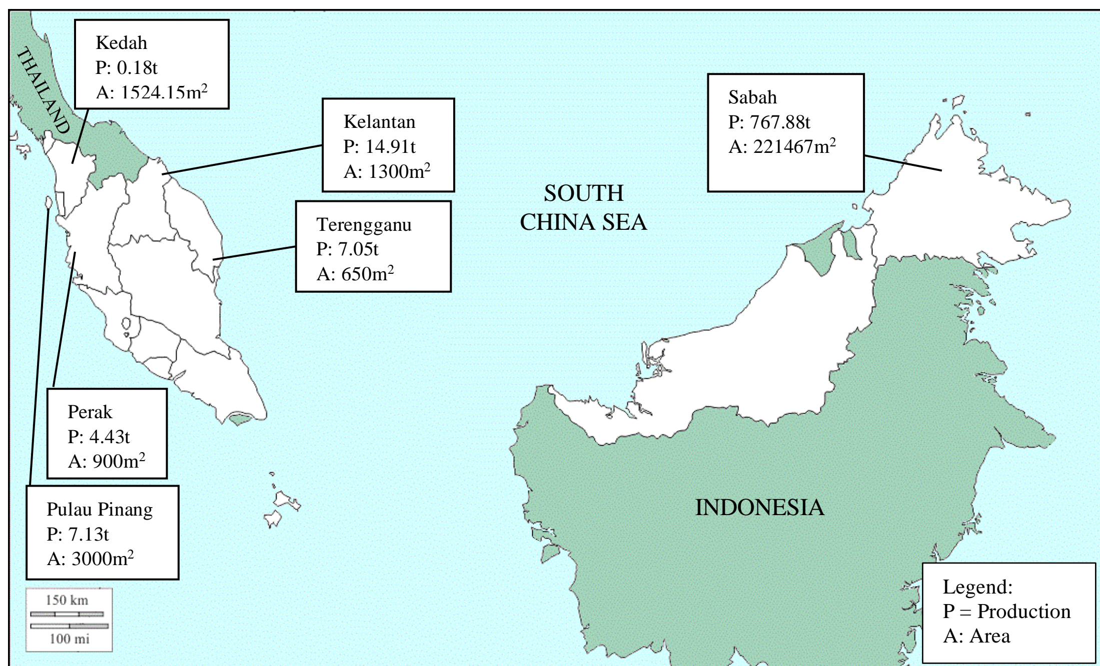

Figure 2.1: Oyster production and production area by states in the year 2016 (Adapted from the Department of Fisheries, 2016).

These oysters are grown along the 45 km long Merbok River where mangrove forests thrive and there are approximately 60,000 oysters being farmed on the floating raft at one time (Figure 2.2). From the successful KTP pilot project in Kedah, the programme is now replicated in other parts of Malaysia such as local communities in Perak, Selangor and Johor. The retail prices of oysters reported by the Department of Fisheries (2016) ranged from RM 10,000/ metric tonne (Sabah) to RM 29,966/ metric tonne (Perak).

Figure 2.2: Baskets containing tropical oyster agitated on rafts in a Kedah Knowledge Transfer Programme site (Source: Loh, 2016).

There are only two known commercial oyster hatcheries in ASEAN, one in Vietnam and one in Penang, Malaysia (set up in 2009) (Tan *et al.*, 2014). The remaining ASEAN countries heavily rely on natural spatfall and imported seeds. There was an increase in oyster production from year 2005 to 2010 (Figure 2.3). The production of edible oysters in Penang rose from 6.84 metric tonnes in 2005 to 7.12

metric tonnes in 2010, which might have been contributed by the rise in seed production by the local hatchery.

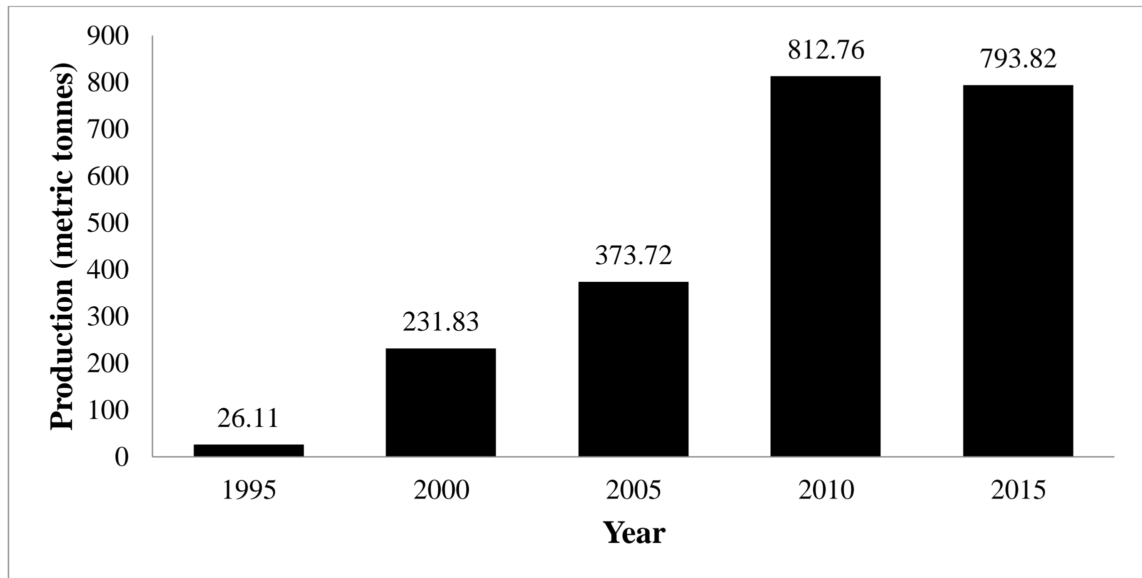

Figure 2.3: Oyster Production in Malaysia from Year 1995 to 2015 (Department of Fisheries Malaysia, 2016).

Prior to 2012, oyster seeds production was not reported by the Department of Fisheries Malaysia. The private oyster hatchery set up in 2009 remained the only source of constant edible oyster seed supply in Malaysia until 2012, where the government started a hatchery in Pulau Sayak, Kedah. However, the private oyster hatchery was the only one able to supply increasing number of seeds yearly (Department of Fisheries Malaysia, 2012).

2.2 Oyster species cultured in Malaysia

In the ASEAN region, the most commonly farmed oyster species are *Magallana belcheri* (Sowerby, 1871), *M. bilineata* (Röding, 1798) and *Saccostrea glomerata* (Iredale & Roughley, 1933) (Sahavacharin, 1995; Bussarawit & Cedhagen, 2012; Li *et al.*, 2017). The oyster species cultured in Malaysia (Plate 2.1) are mainly

true oysters from the Ostreidae family. According to Tan *et al.* (2014), the two-major species farmed are *M. belcheri* and *M. bilineata* where both are mostly presented in half-shell form for seafood restaurants and seafood buffet lines. Another important species, the *Saccostrea cuccullata* is mainly sold in shucked form at local markets mainly for a hawker stall favourite, oyster omelette, and are harvested from intertidal areas.

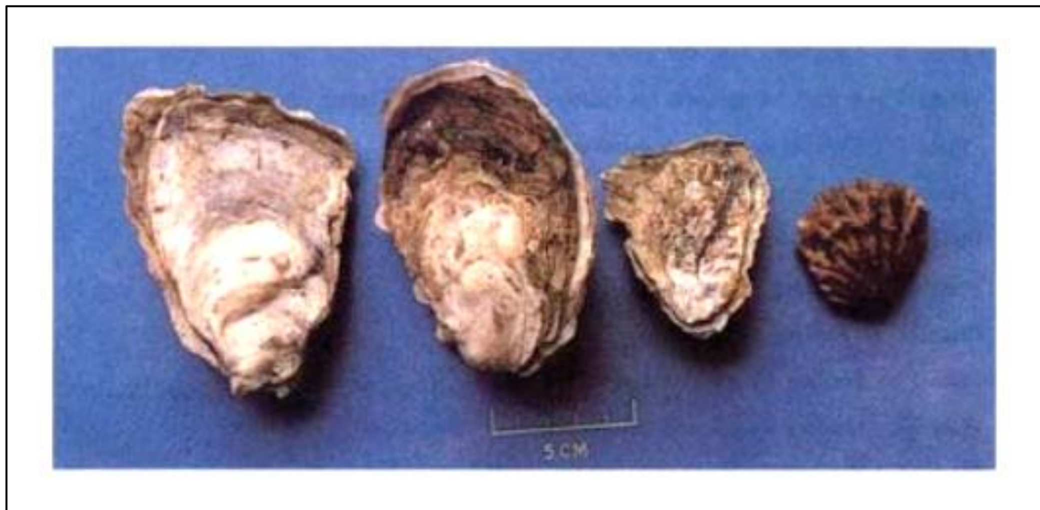

Plate 2.1: Commercially important oyster species in Malaysia from left to right. *Magallana bilineata, M. belcheri, Saccostrea* spp., *Dendostrea folium* (Adapted from Yatim (1993)

Compared to the global oyster meat and processed products market, Malaysia demands a smaller niche for oyster products. In other countries, the oyster flesh are processed in various forms such as shucked, brined, dried, fried, smoked, canned, frozen (Walton *et al.*, 2013; Featherstone, 2016) or made into oyster by-products such as oyster sauce (a very popular Asian cooking condiment and seasoning due to its high glutamate content which brings about umami flavour) (Yoshida, 2009; Nguyen & Wang, 2012; Smith, 2015).

Compared to its oyster producing neighbours in the ASEAN region, Malaysia has one of the lowest aquaculture productions, which is even lower than Indonesia's capture fisheries production (Figure 2.4). Collectively as a region, ASEAN contributed 1.34% to the global total oyster production in 2015 (comparable to France's Pacific oyster production in the same year) (FishStatJ, 2018). Nevertheless, there is plenty of room for rapid expansion to supply the world's demand for the marine shellfish.

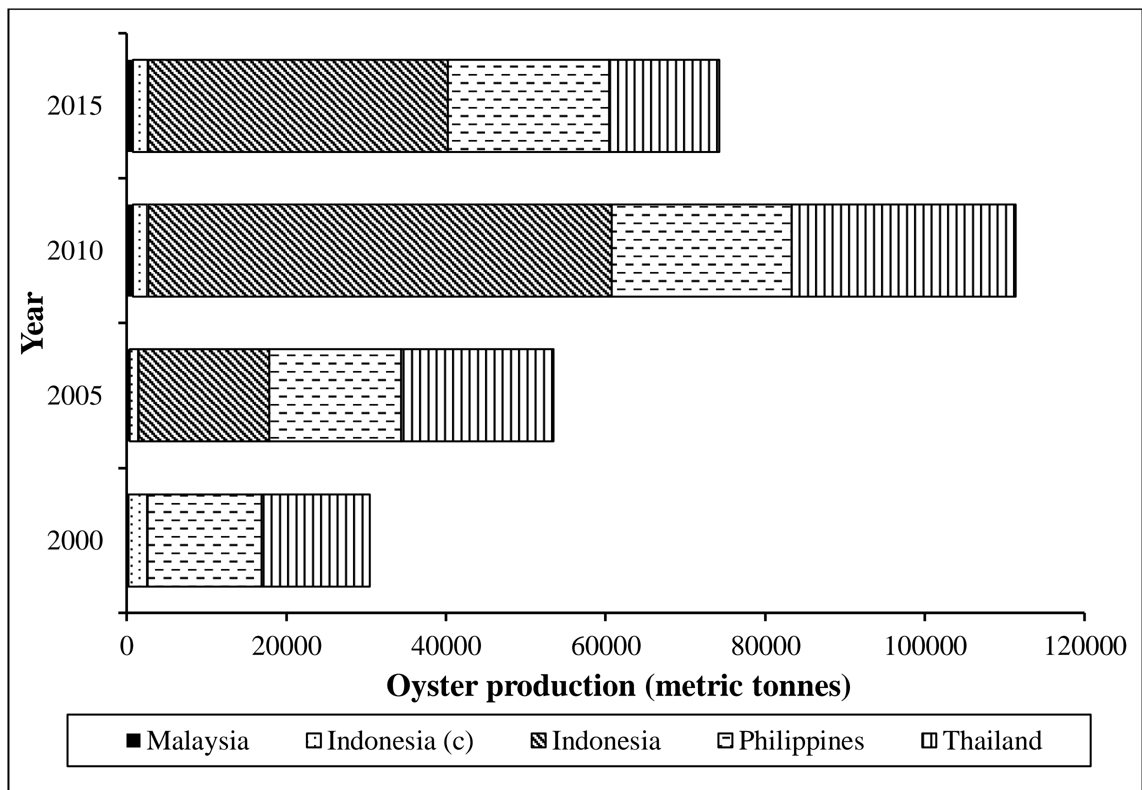

Figure 2.4: Aquaculture and capture (c) oyster production in ASEAN countries (adapted from FishStatJ, 2018).

2.3 *Magallana bilineata* as the chosen study species

Magallana bilineata was chosen as the study species because it is a commercially farmed species of oyster cultured in Malaysia. In earlier larval development, *M. bilineata* can withstand a larger range of salinities (15-30 ppt) to

develop from fertilised eggs to normal one day old D-larvae (Ng *et al.*, 2016) compared to *M. belcheri* (24-30 ppt) (Tan & Wong, 1996). As the culture of tropical oysters is generally assumed to be within coastal tropical temperatures of 22 and 33°C (Ooi, 2018), limited studies have been carried out to assess temperature tolerance of the oysters under 20°C. *Magallana bilineata* larvae is known to be able to survive in 20°C but growth was higher in higher temperatures (Teh *et al.*, 2016). *M. gigas*, a temperate oyster species can survive in 17°C but similarly shows higher growth, metamorphosis and settlement success in higher temperatures (Rico-Villa *et al.*, 2009). Hence, little is known about the ability of *M. bilineata* to withstand temperatures under 20°C or its survivability in cryogenic temperatures.

2.4 Constraints of oyster culture in the natural habitat

Overall, oysters are known to tolerate a wide range of environmental parameters such as salinity and temperature, having existed in marine or brackish habitats with daily fluctuations that come in with the tide (Sudrajat, 1990; Langdon & Robinson, 1996; Sudradjat, 1996; Mann *et al.* in Shatkin *et al.*, 1997; Devakie & Ali, 2000; Huo *et al.*, 2014; FAO, 2018). The reproductive cycle of the tropical oyster is regulated by tropical seasonality of the region, with some degrees of synchronization between male and female spawning. (Paixão, 2013). Unlike tropical oysters, temperate oysters such as *M. gigas* has a defined maturation and spawning period, which is regulated by seawater temperature and usually matures and spawns within the months of April and August (Dridi *et al.*, 2014). Studies have shown that tropical oyster, *Crassostrea corteziensis* in Mexico experienced stocks with mature females without accompanying mature males up to two months at a time (Rodríguez-Jaramillo *et al.*, 2008) and *C. tulipa* males in Brazil peaking 100% in the dry-rainy transition period

and females only 73.37% with no mature males in dry season (from October to December 2010) (Paixão, 2013).

Oyster hatcheries set up to supply oyster seeds to boost oyster farming still mainly depend on wild stock. Variations in the gonadal condition during the tropical rainy and dry season transitions may pose a possible threat to Malaysian oyster culture as gonadal condition at harvest remains uncertain, like the realities of other tropical oyster species worldwide. Synchronized mature broodstock unavailability will cause a disruption in continuing the life cycle, which could potentially be the downfall of the continuity of the industry. Seed stocking alternatives as a viable seedstock solution should be explored.

2.5 Cryopreservation as a solution

Cryopreservation refers to suspended animation of structurally intact living cells by subjecting the samples to very low temperatures, without altering its natural biological mechanism after thawing (Hassan *et al*., 2015; Pegg, 2015). Cryopreservation serves well in animal conservation to preserve specific strains of interest or species (Prentice & Anzar, 2011) and to allow unlimited fry production to boost commercial hatcheries (Hu *et al*., 2013).

The first documented successful cryopreservation is in frog sperm (Luyet & Hodapp, 1938) and human sperm (Jahnel in Isachenko, Rahimi, Mallmann *et al.*, 2011). It was not until 1949 that Polge *et al.* used glycerol as a cryoprotective agent for human sperm that sprung the field of cryobiology into action. In terms of aquatic species, the Pacific herring sperm was the first reported success in 1953 (Blaxter, 1953). The field of marine invertebrate cryopreservation is less-developed compared to that of freshwater fish cryopreservation but has nonetheless produced studies on 50 species

comprising of sea urchins, oysters, abalones, corals, clams, mussels, starfish, rotifers, sand dollar, sea cucumber, anemone, annelids and barnacles (Paredes, 2015).

Since Lannan's success in 1971, there have been more than 50 published studies on oyster cryopreservation (Hassan *et al*., 2015). As of 2015, 39% of published invertebrate cryopreservation work focused on eleven oyster species. From the total, 64% of the research was carried out on the temperate species, *Magallana gigas*, while only 1 study was carried out on the tropical species, *M. bilineata* (Paredes, 2015). The remaining 34% of oyster cryopreservation studies were carried out on *C. angulata, C. rhizophorae, C. tulipa, C. virginica*, *Ostrea chilensis, O. edulis, Pinctada imbricata, P. margaritifera* and *Saccostrea cuccullata*. Most cryopreservation studies on oysters were conducted on Pacific oyster, *Magallana gigas* sperm (Gwo, 2001; Hassan *et al*., 2015). Typically, the sperm is chosen because they are smaller and therefore having a larger surface area to volume ratio and a higher rate of water cryoprotectant movement into and out of cells, lessening ice formation at freezing temperatures (Anchamparuthy *et al*., 2009). Technically, the most sensitive process is the lowering of temperature surrounding the cells (Yawn, 2014).

2.6 Cryopreservation techniques

Cryopreservation involves manipulation of several factors which are key to successful preservation such as types of cryoprotecting agents (CPA) and sperm extenders, variable cooling and thawing protocols as well as storing agents (Stacey & Day, 2007). The procedure varies among species.

2.6.1 Cryoprotecting agents (CPA)

A cryoprotecting agent is usually a high concentration solute capable of driving the movement of water out of a cell and increasing solutes within a cell, thus limiting internal cellular ice formation in low temperatures (Best, 2015; Joshi, 2016). CPA is typically characterized into two groups which are the permeable CPA and non-permeable CPA. Equilibration time is needed to ensure CPA penetrate the cells or equilibrate the surrounding solutes. It is generally kept to a minimum to avoid sperm exhaustion from dilution. Since the sperm is small, an equilibrium time of about 10 minutes is sufficient (Noble, 2003).

2.6.1 (a) Permeable cryoprotecting agents

A permeable CPA is a soluble compound with relatively low molecular weight (<100g/mol) (Karow, 1987) and works by first starting out as a hypertonic medium which gradually draws water out from inside the cells due to osmotic pressure difference and at the same time the CPA will partially replace the space in the cell. At equilibration, the cell would have regained its original volume. Commonly used permeable CPAs are dimethylsulfoxide (DMSO), glycerol, methanol, ethylene glycol, propylene glycol, formamide and butanediol (Best, 2015). For oyster cryopreservation, the most commonly used CPA is DMSO due to its low toxicity and sufficient protectant ability (Ieropoli *et al*., 2004). Despite its toxicity to *C. rhizophorae* sperm and oocytes, glycerol was shown to be least toxic to its embryos (Sansone *et al*. 2005). Glycerol was also studied on *M. gigas* sperm, but it proved to cause higher cell damage compared to DMSO (Park *et al*., 2013). The undocumented use of Glycerol on *M. bilineata* is therefore worth a trial to fill in the knowledge gap in oyster cryopreservation.

2.6.1 (b) Dimethylsulfoxide (DMSO)

Dimethylsulfoxide is one of the most widely used permeable CPA in cryopreservation and has a molecular weight of 78.13g/mol. It has one of the highest fertilization percentages with thawed oyster sperm was at concentrations between 4-20% (Adams *et al*., 2004; Yang *et al*., 2012). The DMSO structure $(CH_3)_2SO$ contains two hydrophobic methyl groups and a polar hydrophilic sulfoxide group. Its sulfoxide group could form hydrogen bonds with lipids in the plasma membrane while the methyl groups interact with proteins and lipids to induce a rearrangement of plasma membrane. This action increases membrane fluidity, thus enhancing survivability during cryopreservation processes (Holt, 2000; Best, 2015).

DMSO was found to be present in the aquatic environment by Andreae (1980) in ocean surfaces, rivers and lake at concentrations >10nmol/L and is not detected in the euphotic zone. DMSO is a product of phytoplankton activity whereby dimethysulfide (DMS) is oxidised to DMSO photochemically (Brimblecombe & Shooter, 1986) or by microbial degradation of phototrophic bacteria (Zeyer *et al*., 1987). It was first synthesized in 1866 by Alexander Zaytsev and was used in on bull spermatozoa by Lovelock & Bishop in 1959 (Jang *et al*., 2017). It's first application on oysters was reported to yield 10.3% fertilized ova with DMSO 20% cryopreserved sperm.

2.6.1 (c) Glycerol

Glycerol is a permeable CPA with a molecular weight of 92.09g/mol. The glycerol structure $C_3H_5(OH)_3$ contains three hydroxyl groups, making it hygroscopic. It forms hydrogen bonds with surrounding water molecules, making ice crystal formation difficult (Bhattacharya & Prajapati, 2016). In nature, glycerol is produced

naturally by marine yeast (Hernández-Saavedra *et al*., 1995) as a response to increase salinity, mainly to maintain internal osmotic potential. The rainbow smelt (*Osmerus mordax*) has also been known to produce plasma glycerol (levels approaching 500 mmol l^{-1}) in the liver when triggered by low temperature of -1°C (Driedzic *et al*., 2006), enabling it to flourish and naturally cryoprotect itself in -1.8°C frozen sea water. It sustains the glycerol levels through dietary carbohydrate and protein intake (Driedzic, 2015).

Glycerol was discovered by Carl Wilhelm Scheele in 1779 as it washed out from a mixture of heated lead oxide and olive oil. Its source today is as a by-product from manufacturing soap (Newman, 1968). In cryopreservation, the use of glycerol proved to be more frequent in *Xiphophorus* (fish) sperm than in oysters. The most effective concentration of glycerol in retaining motility of *Xiphophorus couchianus* (Huang, Dong & Tiersch, 2004) and *X. helleri* sperm (Huang *et al*., 2004 a, b) is glycerol 14%. When studied in *C. rhizophorae*, glycerol was the most toxic to sperm and oocyte with half maximal effective concentration after 24 hours (EC_{50} – 24hr) percentage mean values of 2.07% and 3.46%, respectively. However, the inverse was true when tested on trochophores, where glycerol was the least toxic among all CPA (Sansone *et al*., 2005).

2.6.1 (d) Non-permeable cryoprotecting agents

A non-permeable CPA is a soluble compound with relatively high molecular weight, which draws out water from the cell but the compound itself remains in the extracellular solution surrounding the cell. Some non-permeable CPAs are from the groups of carbohydrates, polymers, polyols, polysaccharide, amines, proteins and phospholipids. Sugars are an inexpensive choice of CPA to study and it acts as an

osmotic buffer to reduce osmotic shock resulting from dilution of CPA after cryostorage (Isachenko, Isachenko, Sanchez *et al.*, 2011; Hubel, 2018). For oyster cryopreservation, non-permeable CPA is used alongside permeable CPA to reduce its toxicity while maintaining its effectiveness to draw water out from the cell (Hassan *et al.*, 2015).

2.6.1 (e) Glucose

Glucose is an almost non-permeable CPA with a molecular weight of 180.15g/mol. It has a structural formula of $C_6H_{12}O_6$ and is found to be of low efficiency in CPA activity (Lovelock, 1954). However, glucose has been shown to reduce body ice content and cryoinjury in wood frog (*Rana sylvatica*) erythrocyte (Costanzo *et al.*, 1993). The natural cryoprotecting ability is determined by the size of hepatic glycogen reserve as the amount of glucose is produced via liver glycogenolysis triggered by low temperatures in winter (-3°C) (Steiner *et al.*, 2000; Dinsmore II & Swanson, 2008).

Glucose was first isolated by Andreas Marggraf in 1747 from raisins. Glucose is the building block for all starches and carbohydrates and can be produced via photosynthesis from water and carbon dioxide, in the presence of sunlight. Commercially, glucose is produced from cornstarch and that undergoes acid-enzymatic hydrolysis (Scallett & Ehrenthal, 1967). Its use as a CPA in shellfish cryopreservation was effective at 0.5M when used with permeable CPA ethylene glycol on surf clam *Spisula sachalinensis* umbo larvae (Choi *et al.*, 2008). In the case of *M. gigas*, glucose at 0.2M and 0.5M were found to be more effective in protecting umbo larvae when used with ethylene glycol than with DMSO, like in the study with surf clam (Choi & Chang, 2014).

2.6.1 (f) Sucrose

Sucrose is a non-permeable CPA with a molecular weight of 342.29g/mol. It has a structural formula of $C_{12}H_{22}O_{11}$ and is made up of one glucose and one fructose molecule. Though it is not produced in animals for cold resistance, it is produced during cold acclimation of *Colobanthus quitensis* and *Deschampsia antartica*, the only two vascular plants to colonize the Antarctic (Bravo *et al.*, 2001).

Sucrose was discovered by Andreas Marggraf in sugarbeets in 1747 and an industrial process for sucrose extraction was invented by Franz Achard in 1802 (White, 2014). It is commercially produced from sugarcane and sugarbeets which undergo juicing to extract the natural sucrose. Concentration of the raw sucrose juice produces syrups and crystalized sucrose (Eggleston, 2008). Its use as CPA in shellfish cryopreservation was proven to be most protective (96.1±1.0% survival) at 0.2M when used with permeable CPA DMSO and ethylene glycol on surf clam *Spisula sachalinensis* umbo larvae (Choi *et al.*, 2008). Sucrose 0.2 M and 0.5M used with ethylene glycol produced the highest survival in *M. gigas* umbo larvae, compared to DMSO (Choi & Chang, 2014).

2.6.2 Extenders

Sperm extenders are commonly used to aid post-thaw sperm viability as pure semen alone is usually not suitable for freezing (Scott & Baynes, 1980; Suquet *et al.*, 2012). Sperm motility of some fish species lasts between 2 to 20 minutes (Cosson *et al.*, 2008a) and is brought on by hypotonicity (for freshwater fish) and hypertonicity (for marine fish) (Cosson, 2004; Cosson *et al.*, 2008b). Therefore, the main usage of a sperm extender is to suppress the initiation of sperm motility during handling and storage (Orfao *et al.*, 2011; Chapman, 2016). In these fish species, the extender

composition mimics the osmolality of the testis and seminal plasma where sperm are immotile (Ladoktha *et al*., 2015).

The opposite is true for marine oysters whose sperm movements range from several hours to days and extenders in this case, provide an isotonic environment which lessens sperm deformities (Hassan *et al*., 2015). The sperm motility of marine bivalves such as Pacific oyster, *M. gigas* is triggered by alkaline pH (Suquet *et al*., 2012) and inhibited at pH 7 (Alavi *et al*., 2014). In marine oysters, the extender used mainly consist of a saline solution such as filtered seawater (Vitiello *et al*., 2011) or a balanced salt-solution with the addition of sugar and pH buffers (Yang *et al*., 2012; Yang *et al*., 2015).

It is recommended that sperm to extender ratio for *C. virginica* be limited to 1:1 or 1:3 if the sperm is to be kept for more than 24 hours. Otherwise, undiluted sperm would be a better choice for higher fertilizing ability. It is also shown that high sperm to extender ratio (1:31) produced the lowest motility. Paniagua-Chavez *et al*., 1998). Equilibration time is needed to ensure surrounding salts and sugars in surrounding solutes equilibrate with the internal solutes of the sperm. It is generally kept to a minimum to avoid sperm exhaustion from dilution. Since the sperm is small, an equilibrium time of about 10 minutes is enough (Noble, 2003).

2.6.3 Containers for freezing

Containers for biological specimen containment can come in the shape of vials or straws (Figure 2.5), usually made with specially formulated polypropylene to withstand very low temperatures of under -196°C. The volume of the vials or tubes determine cooling and thawing rates and usually, a small volume straw or 0.25ml or 0.5ml is used (Tiersch *et al*., 2007).

Figure 2.5: Storage containers for cryopreservation (Source: Worthington Industries)

Commercial cryopreservation of oyster sperm employs a computer-controlled system to automatically fill, seal and label the straws (Yang *et al.*, 2012). Cryovials are more convenient and can be reused, compared to straws which will be broken to release the thawed sperm afterwards (The Jackson Laboratory, 2018.). A liquid nitrogen tank is usually used to store frozen samples and can be refilled when needed. The container choice greatly depends on the storage system set up in the lab.

2.6.4 Freezing process

Generally, there are two types of freezing methods for cryopreservation, which are the controlled-rate freezing and non-programmable freezing method. The former can involve a single (Yang *et al.*, 2012) or multiple freezing steps (Ieropoli *et al.*, 2004) with variable rates and generally costs more to set up. The non-programmable freezing method have uncontrollable freezing rates as it is affected by the distance of the sperm sample from the cold source (liquid nitrogen vapour (Smith *et al.*, 2012; Liu *et al.*, 2014a, b), ethanol-dry ice bath (Santos *et al.*, 2017), methanol-dry ice bath (Adams *et*

al., 2009)), exposure time, volume of the sample and container type, but is generally easier to handle and is more cost effective (Li, 2012).

2.6.5 Thawing process

Prior to fertilization of cryopreserved biological specimens, a thawing procedure is needed. Generally, the thawing time is quick and shorter than the cooling or freezing time. This ensures that recrystallization of internal ice is prevented when the solutes approach their freezing point (Mazur, 2004). Thawing temperature and time ranges from 16°C to 75°C and 2 seconds to 2 minutes, respectively (Hassan *et al*., 2015). The thawing time is hard to estimate (Tiersch, 2011) and is usually done by visual inspection. Thawing is completed when specimen in the container appear liquid.

2.6.6 Current practice of oyster sperm cryopreservation techniques

The current practice of oyster sperm cryopreservation generally begins with the pooling of oyster sperm from several individuals and suspending the pooled sperm in an extender, before being inserted into a cryogenic container of choice such as the cryogenic straw or vials. (Hassan *et al*., 2015; Chapman, 2016; Santos, 2018). Then, the sperm is let to equilibrate in permeable or non-permeable cryoprotectants, or a combination of both for a period, before being cooled in a single step or multiple cooling steps, usually ending in immersion into liquid nitrogen. (Dong *et al.*, 2005; Dong *et al.,* 2007; Choi & Chang, 2014; Hassan *et al*., 2017). The frozen sperm is then taken out of the liquid nitrogen and fertilized with oyster oocytes (Ieropoli *et al*., 2004; Labbé *et al*., 2018).

CHAPER 3

MATERIALS AND METHODS

3.1 Gamete collection

3.1.1 Broodstock preparation

Magallana bilineata oysters were bought from Sungai Merbok oyster farm, which is a mangrove fringed estuary situated in Kedah state, in Malaysia. The Sungai Merbok estuary displays a pronounced fortnightly neap-spring stratification-destratification cycle and generates brackish water with salinity levels of between 23.5 to 30 psu (Ong *et al.*, 1991; Muhammad Syukri (2009). The oysters were cleaned by scrapping and brushing. Biofoulers such as barnacles and tube worms were scrapped off and sediments were brushed off (Plate 3.1). Oyster cleaning is essential to minimize specimen contamination, which might cause sperm and oocyte concentration estimation to be difficult. Specimen contamination could also lower the chances of fertilization.

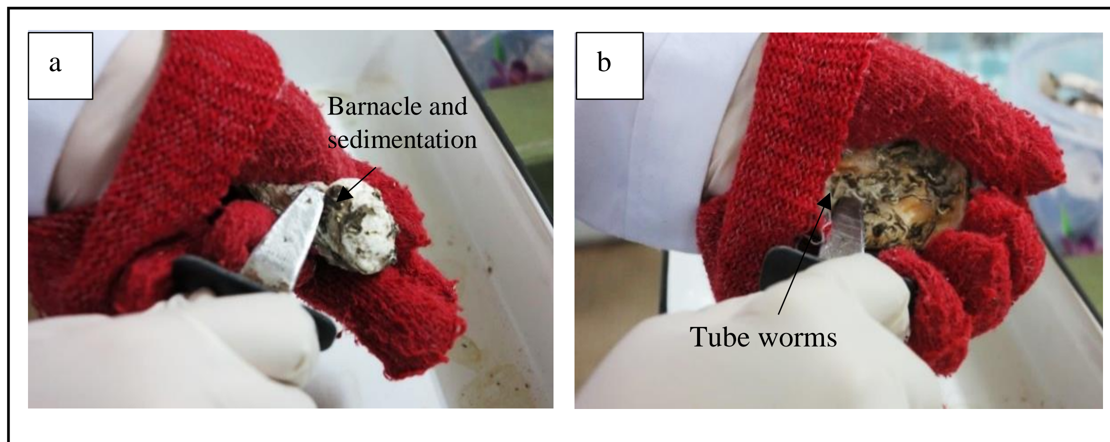

Plate 3.1: Scrapping biofoulers before opening the oyster; a. Barnacle and sedimentation, b. Tube worms.

The oysters' length and width were measured with a digital vernier caliper (Gere Precision Sweden) according to Figure 3.1 and the oysters' weight were measured with a digital scale (Shimadzu ELB3000). The measurement of each individual is recorded and averaged to get the mean.

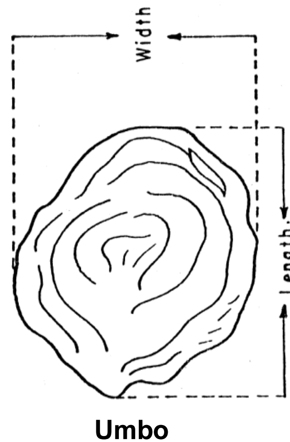

Figure 3.1: Oyster shell measurement method used in this experiment (Warren, 1958)

The experiment used gametes from sexually mature oysters with ripe gonads (Plate 3.2) and gametes from spawned oysters were not used due to its low fertilization potential. The sexually mature oysters were differentiated from the non-sexually mature oysters by the presence of obvious gonadal development which is cream-coloured and opaque, covering a majority of the visceral mass, whereas the non-sexually mature or spawned oysters have little or close to none and show a more translucent visceral mass membrane, with the gastro-intestinal area more visible. Such exclusion was necessary to lessen the fertilization ability variation between experiments of different batches.

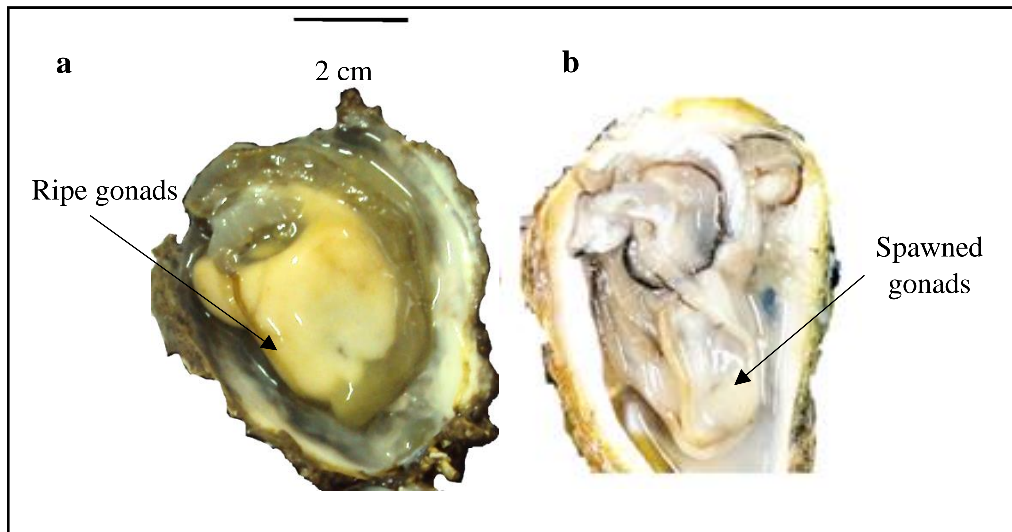

Plate 3.2: Gamete condition of oysters; a. Mature oyster, b. Spawned oyster.

The sex of the oysters was determined by using a small portion of gamete scrapped from the gonad and was then examined under the microscope (Plate 3.3). The scrapper was washed after examining every oyster to reduce accidental fertilization.

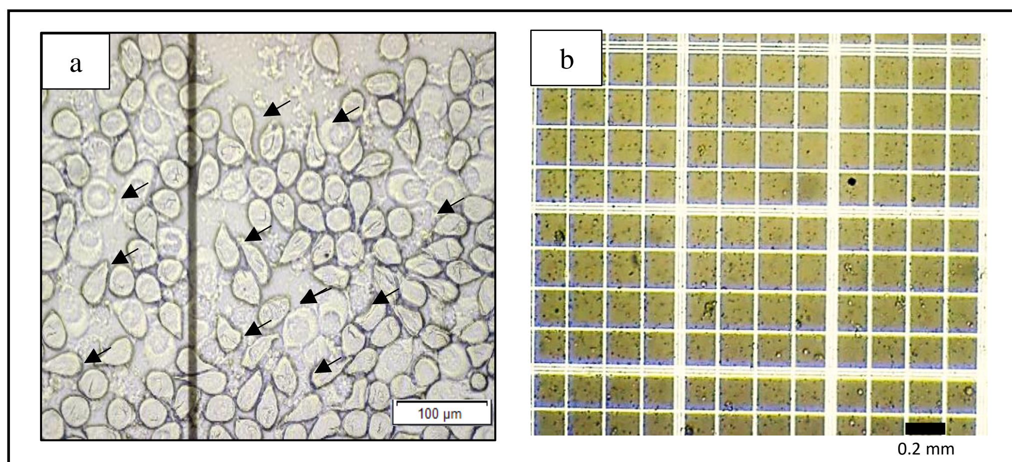

Plate 3.3: Determination of sex of oysters under the microscope at 200x magnification; a. black arrows show a smear of tear drop shaped oocytes, indicating a female oyster, b. a smear of oyster sperm showing the sperm heads as black spheres, indicating a male oyster.