Reproductive strategy and sperm cryopreservation of a spermcasting bivalve - the Australian flat oyster *Ostrea angasi*

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by

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Abstract

Spermcasting is a unique mode of reproduction in which males release gametes in water and females acquire male gametes and fertilize eggs inside the mantle cavity. Although spermcasting occurs in variety of sessile species, this spawning strategy is the least known among other reproductive modes in marine species. Some spermcasting bivalves are also important species in aquaculture but the breeding programs of these species are hindered due to the poor understanding of spermcast spawning and lack of techniques to control reproduction. This thesis aims to (a) elucidate adaptive strategies in the reproduction of a spermcasting species - the Australian flat oyster *Ostrea angasi*, (b) develop techniques for its sperm quantification, and (c) develop protocols sperm cryopreservation. Five experiments were conducted to achieve the thesis aims.

In Experiment 1, the structure and functional properties of male gametes were investigated to understand the reproductive strategy in *O. angasi*. The male gametes of this species are released in a spermatozeugma where a cluster of sperm is bounded by a gelatinous membrane. In seawater, individual sperm swim off the spermatozeugmata by dissociating the membrane. The sperm of *O. angasi* have one more mitochondrion than broadcasting oysters although their sperm dimensions are similar. The duration of spermatozeugmata dissociation and sperm motility varied among individuals of different masculine levels. The hermaphrodites with a large proportion of male gametes could maintain spermatozeugmata integrity and sperm motility longer than those with a small proportion of male gametes. Spermatozeugmata structure and functional properties have adaptive significance in gamete dispersal in seawater and fertilization success in the female mantle cavity.

In Experiment 2, adaptive strategies in gametogenesis, sex ratio and energy metabolism were studied to further understand the advantages of spermcast spawning in *O. angasi*. During gametogenesis, different stages of spermatozeugmata were found within a male

or hermaphroditic oyster while oocytes mature simultaneously within a female or hermaphroditic oyster. The histological observation of partially spawned hermaphroditic oyster indicated that spermatozeugmata would be released before egg ovulation. In the sex ratio analysis of 2-3 years old *O. angasi*, the percentage of male, female, hermaphrodite and undifferentiated sexes was 41.3%, 5.8%, 46.7% and 6.2%, respectively. Glycogen was the main energy source for gametogenesis. The periods of energy storage versus energy utilization overlapped, and *O. angasi* displayed an intermediate energy metabolism strategy between conservative and opportunistic species. The patterns in gamete development, sex ratio and energy metabolism of *O. angasi* are of significance to reproductive and physiological adaptations in spermcasting species.

In Experiment 3, a rapid, cost-effective and reliable technique for estimation of sperm concentration was developed with spectrophotometry. A regression model of $y = 1\times10^{-8} x + 0.163$; $r^2 = 0.996$ was generated at 350 nm wavelength. The model was validated by comparing sperm counts with the haemacytometer method. The spectrophotometric technique would increase the efficiency of sperm concentration determination and facilitate breeding programs and cryopreservation.

In Experiments 4 and 5, sperm cryopreservation protocols were developed by using programmable and non-programmable freezing methods. Sperm motility and plasma membrane integrity were used as indicators to assess sperm quality. The highest post-thaw sperm survival was achieved by the non-programmable freezing method, with the protocol including sperm equilibration with 15% ethylene glycol + 0.2 M trehalose for 20 min, package in 0.25 ml straws, exposure to liquid nitrogen vapor for 10 min at 8 cm above the liquid nitrogen surface, and storage in liquid nitrogen. This protocol would

open a new option for effective cryopreservation to assist the development of controlled breeding and genetic improvement programs.

This thesis provides new knowledge to understand the reproductive strategy and physiological adaptation of the spermcasting oyster *O. angasi*. The regression model established with spectrophotometry provides a rapid method to quantify sperm concentration in this species. The newly-developed sperm cryopreservation protocol could overcome the seasonal constraints in sperm supply and provide a year-round superior sperm stock for breeding programs. The research outcomes of this thesis would enhance the capacity and efficiency for development of breeding programs in spermcasting molluscan species.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Md Mahbubul Hassan

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Chapter 1: General Introduction

1. General Introduction

The marine environment supports a variety of life forms with diverse spawning modes to maximize reproductive success. While most marine species release gametes into water column, some species transfer sperm to females directly by copulatory spawning. Interestingly, some sessile species have a unique spawning mode, termed as spermcasting, in which sperm are acquired by females to fertilize eggs in their body cavity. Spermcasting is involved in diverse taxonomic groups including hydroids, corals, polychaetes, bivalves, tunicates, entoprocts, brachiopods, bryozoans and pterobranchs (Pemberton et al., 2003, appendix B). The fertilization dynamics in spermcasting species are different from broadcasting species. Sperm dilution in seawater often causes sperm limitation resulting poor fertilization rates in broadcasting species (Yund, 2000), but spermcasting species can achieve similar fertilization rate compared with broadcasting species at a sperm density of two-three fold lower (Bishop 1998; Pemberton et al., 2003).

Some spermcasting bivalves greatly contribute to economy through aquaculture. For instance, oysters of the genus *Ostrea*, commonly known as flat oysters, are commercially farmed worldwide including *O. edulis* in Europe, *O. chilensis* in Chile and New Zealand, *O. angasi* in Australia, and *O. lurida* in the USA. One of the major obstacles for the further development of flat oyster aquaculture is the occurrence of bonamiasis; a serious parasitic protozoan disease affecting oyster survival (Engelsma et al., 2014; Kissner et al., 2014). Selective breeding to develop an oyster strain resistant to *Bonamia* is considered an effective approach to address the flat oyster mortality associated with the bonamiasis infection (Lynch et al., 2014) as this genetic approach has been applied in many farmed shellfish and finfish species (Piferrer et al., 2009). However, the development of a selective breeding program in spermcasting flat oysters

is constrained by their unique reproductive mode. In artificial reproduction of broadcasting bivalves, the fertilized eggs and larvae are incubated in seawater by maintaining water quality parameters at the optimal range. However, artificial fertilization leading to embryo and larval incubation outside the maternal body is difficult to achieve in spermcasting bivalves as females carry fertilized eggs inside the shells for 2-3 weeks and release the larvae in the water column at a late stage.

In this study, the Australian flat oyster *O. angasi* has been chosen as a representative of spermcasting bivalves to improve the understanding of adaptive strategies in spermcast spawning and develop techniques to assist artificial reproduction. This species was abundant along the Australian south coasts in the late 18th and the early 19th century, but almost disappeared from the natural habitat due to overfishing (Alleway & Connell, 2015). In recent years, this species has been emerging as a potential aquaculture candidate with a gradual increase in farm production since the last decade (O'connor & Dove, 2009).

1.1. Definition of broadcasting and spermcasting in reproductive biology

Considerable ambiguity exists in the literature for terminology in reproductive modes among marine species. For example, the term ‘free spawning’ has been used to describe sperm release without considering egg release or retainment (Levitan, 1998; Yund, 2000; Santelices, 2002), whereas this term has also been used synonymously as broadcasting (Pemberton et al., 2003; Bishop and Pemberton, 2006). In this thesis, a species is considered a broadcast spawner when both male and female release gametes in seawater, and fertilization occurs in the ambient environment. In contrast, a species is referred as a spermcast spawner when male gametes are released in seawater, but maternal adults inhale male gametes to fertilize eggs inside the female body.

1.2. Spermatozeugmata structure and dissociation

Spermcasting species release spermatozeugmata where sperm heads are bounded by a gelatinous membrane and tails extend outside (Maiorova & Adrianov, 2005; Falese et al., 2011). Spermatozeugma has been synonymously termed as a ‘sperm-ball’ (Coe, 1931) or a ‘sperm sphere’ (Ishibashi et al., 2000). In seawater, sperm swim off the spermatozeugma by disintegrating the membrane, and maintain motility for a certain period. In this thesis, the word “spermatozeugma” is used as a singular noun and the word “spermatozeugmata” is used as plural noun. The structural and functional properties of spermatozeugmata have crucial consequences for recruitment success in spermcasting species. However, the important questions of how the structural and functional properties of spermatozeugmata could affect fertilization and recruitment of spermcast spawners have not been answered.

1.3. Gametogenesis, sex ratio and energy metabolism

Gametogenesis is an important process for reproductive precursor cells to undergo cell division and differentiation and form haploid gametes. Reproductive periodicity and spawning pattern such as synchrony or asynchrony are determined by the gametogenesis process. Since sessile bivalves are incapable of moving to find a mate during reproduction, the gametogenesis pattern has a great impact on fertilization success.

The sex changing species may switch to one sex mode to an opposite sex to optimize reproductive output depending on resource and mate availability. Oysters are protandric hermaphrodites but the sex ratio of spermcasting oysters differs from broadcasting oysters. Within a similar size adult individuals, spermcasting oysters have a higher percentage of hermaphrodites than broadcasting oysters (Steele and Mulcahy

1999; Enríquez-Díaz et al. 2009; Acarli et al. 2015). Hermaphroditism is an adaptive strategy in sex changing species to use either male or female function to optimize reproductive success.

Energy allocation for gametogenesis is a fundamental life history trade-off to maximize fitness. Majority of marine bivalve species store energy in different tissues and mobilize energy for gametogenesis, but a few species can metabolize energy for gametogenesis directly from food intake (Mathieu & Lubet, 1993). The energy is stored as glycogen, protein and lipid but the strategy to metabolize each energy component is species-specific. Energy supply from degenerated eggs for the next round of gametogenesis is another strategy to acquire energy (Mendo et al. 2016). The reproductive and physiological adaptations are important aspects in life history for recruitment success and viability in different environments. However, the adaptive mechanisms of gametogenesis, sex ratio and energy metabolism to maximize reproductive success in spermcasting bivalve remain unclear.

1.4. Sperm quantification

Standardization of sperm concentration is essential in breeding programs to optimize genetic variation from multiple males, especially when a specific sperm to egg ratio is required in fertilization (Alliegro & Wright 1983; Gould & Stephano 2003). Sperm concentration is one of the most uncontrolled variables in cryopreservation because optimization of cryoprotectant concentration without a known sperm concentration leads to inconsistent equilibration during freezing (Dong et al. 2007a; Hassan et al. 2015). Haemacytometry is the traditional method for sperm counting (WHO, 1999), but is inefficient for handling a large number of samples especially in family-based breeding programs and cryopreservation. Several techniques such as flow cytometry,

Coulter counter, Makler counter, and computer-assisted sperm analysis have been developed and applied to sperm quality and quantity analysis on human and other vertebrates (Bailey et al. 2007). But due to cost and requirement of highly skilled personnel for handling and maintenance, these techniques may not be practical in breeding programs and sperm cryopreservation when many individuals are involved. The spectrophotometric technique is relatively simple and cheap, and is suitable for rapid sperm quantification (Leclercq et al., 2014), which has been developed (Dong et al., 2005a) and successfully applied in cryopreservation of Pacific oysters (Dong et al., 2005b; Dong et al., 2007b). Although spectrophotometry has useful application, this technique has not yet been standardized for sperm quantification in spermcasting species.

1.5. Cryopreservation

Cryopreservation allows long-term storage of genetic materials without compromising biological functionality. After the revolutionary invention of cryoprotective property of glycerol for human sperm cryopreservation (Polge et al., 1949), this technique has been applied in various purposes including human reproduction (Kuleshova et al., 1999), breeding programs of livestock and aquatic species (Purdy, 2006; Barbas & Mascarenhas, 2009; Martínez-Páramo et al., 2016), and conservation of endangered species (Fickel et al., 2007). Cryopreserved sperm have become a billion-dollar global industry in livestock breeding programs (Tiersch, 2008). In aquatic species, although commercial application of cryopreserved sperm is at infancy, this technique has proven to be very useful in breeding programs (Tiersch et al., 2007). In oysters, sperm cryopreservation research has progressed substantially including application in selective breeding programs (Adams et al., 2008) and high throughput sample processing for

commercial application (Yang et al., 2012). The commercial dairy sperm freezing facilities have also proven to be useful for the Pacific oyster (Dong et al., 2007b). Past sperm cryopreservation research has almost entirely focused on the broadcasting species (Hassan et al., 2015), therefore a considerable knowledge gap exists on cryopreservation of spermcasting species. Since the cryopreservation process can cause cellular injury, optimization of different freezing factors is necessary to develop sperm cryopreservation protocols for new species.

1.6. The spermcasting bivalve *O. angasi*

1.6.1. Taxonomy

The taxonomic details of *O. angasi* are as follows:

Phylum: Mollusca

Class: Bivalvia

Order: Ostreoida

Family: Ostreidae

Genus: *Ostrea*

Species: *O. angasi*

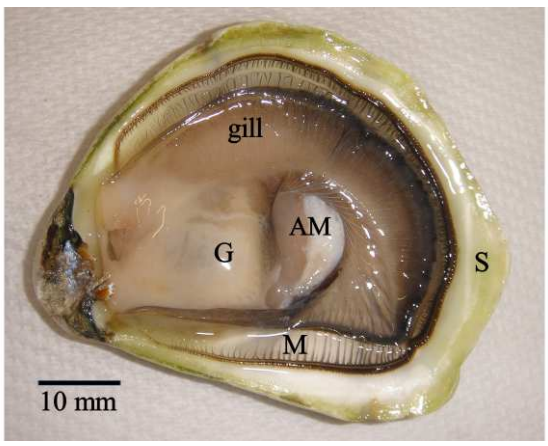

Fig. 1.1: *Ostrea angasi* showing the main internal organs. G, gonad; M, mantle; AM, adductor muscle; S, shell (Photo by MM Hassan).

1.6.2. Fishery history of *O. angasi*

The native Australian flat oyster *O. angasi* has been harvested for centuries as human food, lime and fishhooks. This species used to be an important component of livelihood among coastal aborigines, and the remains can be found in shell middens (Godfrey, 1989). Oyster reefs were explored after European settlement (Peron, 1979) and fishing began by the settlers around 1836, and then flat oysters became the first commercial fishery species in South Australia (Nell, 2001; Kirby, 2004). The oyster beds were distributed over 1500 km coastline in South Australia and dredging occurred at 67 locations where oysters were densely distributed (Inspector of Oyster Fisheries 1892). About 30 sailing cutters were used to dredge oyster beds in one location as a group with iron bars and mesh bags until the catch became low or nil (Wallace-Carter, 1987). The oyster fishery regulators imposed seasonal and localized fishery closure, and enacted regulations since 1853 to control overharvesting. Further regulations were introduced in 1873 to determine minimum dredging size, season, and areas, and penalties for deposition of injurious matter in the oyster bed (Governor, 1873). The licensing system was introduced in 1985 for the commercial harvest (Inspector of Oyster Fisheries, 1886). However, these initiatives were not enough to prevent the resource collapse, and no flat oyster reef is known to exist today in South Australia.

1.6.3. Oyster aquaculture in Australia and farming potential of flat oyster

Oyster farming has a history of over a century and is one of the oldest aquaculture industries in Australia (Nell, 2001). In the last couple of decades, the oyster aquaculture

industry has experienced ups and downs such as the addition of farming areas leading to increased production but also recurrent diseases and implementation of biosecurity measures resulting in reduced overall production. Oyster aquaculture in Australia is mainly based on the native Sydney rock oyster *Saccostrea glomerata* and the introduced Pacific oyster *Crassostrea gigas*. Sydney rock oysters are mainly farmed in New South Wales (NSW) whereas Pacific oysters are farmed in South Australia (SA), Tasmania (TAS) and NSW. Although the initiative of Pacific oyster farming in the mid-20th century was controversial (Medcof & Wolf, 1975), the Pacific oyster industry expanded rapidly. In some growing areas in NSW, the Pacific oyster has outcompeted the Sydney rock oyster due to winter mortality, QX disease and slow growth rate of the latter. Recently, aquaculture of Pacific oyster has formed the basis of oyster industry in the NSW, SA and TAS. The Pacific oyster mortality syndrome (POMS), caused mainly by *Ostreid herpesvirus*1 (OsHV-1), is a threat to the Pacific oyster industry globally. The first outbreak of POMS occurred in France in 2008, and caused nearly total loss of farm stock in the affected areas. In Australia, the first occurrence of POMS outbreak was in 2010 in the Georges River of NSW. In 2013, POMS struck again in Hawkesbury River of NSW and destroyed millions of dollars' farm stocks. In early 2016 the virus was detected in TAS where 80-90% of Pacific oyster spats were produced in Australia. The government immediately banned the translocation of oysters from Tasmania to prevent disease outbreak in other states. Therefore, the entire 53-million-dollar industry is in jeopardy due to the collapse of the spat supply chain.

It is expected that the development of native flat oyster *O. angasi* aquaculture could become one of the solutions for the long-term sustainability of oyster farming in Australia. Because the diseases affecting the Pacific oyster and the Sydney rock oyster do not affect the flat oyster, diversifying the aquaculture with this species has the

potential to minimize the devastating impact of disease on oyster production, investment and employment. Flat oysters are also a premium quality product, and fetch higher market price than other oyster species farmed in Australia, Western Europe and New Zealand. In Australia, the development of flat oyster farming initiated in the 1990's, and has increased gradually in the last decade (O'connor and Dove, 2009). Therefore, the flat oyster has become an emerging aquaculture species in Australia (Heasman et al., 2004), with the development of fast growing and disease resistant strains as one of the key research priorities.

1.7. Objectives

This thesis aims to understand the reproductive strategies in spermcast spawning oysters and develop techniques for sperm quantification and cryopreservation to facilitate the development of breeding programs in spermcasting species. The specific objectives of this thesis are:

(1) To understand the role of structural and functional properties of spermatozeugmata in spawning of spermcasting bivalves;

(2) To reveal the gametogenesis, sex ratio and energy metabolism pattern in *O. angasi* and their implications in reproductive and physiological adaptations of spermcasting bivalves;

(3) To establish a spectrophotometric technique for reliable and rapid estimation of sperm concentration; and

(4) To develop sperm cryopreservation protocol by optimizing key factors that affect the quality of post-thaw sperm.

1.8. Thesis structure

This thesis consists of eight chapters, a general introduction, a literature review, five chapters reporting research results and a general discussion. The literature review

chapter and each research chapter are written as a stand-alone manuscript, and these chapters have been published, accepted for publication or currently under review in peer-reviewed journals. Consequently, some unavoidable repetitions exist among the chapters especially in background and methods.

Although independent objectives are specified in each chapter, all the objectives are complementary to thesis aims and objectives stated in this introduction chapter. Within each chapter, the word 'study' refers to the sole chapter. All the studies were performed by the author of this thesis under the supervision of the principal and the associate supervisor. As such, both supervisors are listed as co-authors for all the manuscripts. In chapter 6, a third co-author contributed to experiment design and data collection. Although manuscripts have been submitted, accepted or published in different journal formats, all the chapters have been reformatted to ensure consistency in the thesis.

Chapters 1 is a general introduction of the thesis that outlines the knowledge gap in the reproduction of spermcasting species and the need to develop techniques to facilitate future breeding programs of spermcasting bivalves.

Chapter 2 addresses the aspects of spawning success in spermcasting *O. angasi* based on spermatozeugmata structure and dissociation (Objective 1). Spermcasting species produce spermatozeugma which is a cluster of sperm bounded by a gelatinous membrane. In seawater, individual sperm swim off a spermatozeugma by dissociating the membrane. Interestingly, the duration of spermatozeugmata dissociation and sperm motility varies depending on the masculinity level in a broodstock. The sperm dimensions of the spermcasting *O. angasi* are similar to the broadcasting species, but have one additional mitochondrion. The implications of these aspects to spawning success of spermcasting species are discussed.

This chapter has been published in Tissue and Cell as:

Hassan, M. M., Qin, J. G., & Li, X. (2016). Spermatozeugmata structure and dissociation of the Australian flat oyster *Ostera angasi*: Implications for reproductive strategy. Tissue and Cell, 48, 152-159.

Chapter 3 addresses the adaptive significance of gametogenesis, sex ratio and energy metabolism in spawning (Objective 2). Gonad histology reveals that spermatozeugmata developed asynchronously but the eggs developed synchronously. The population of *O. angasi* had very high proportion of hermaphrodites and the male vs female ratio was highly skewed. To adapt to energy demand for gametogenesis and physiological maintenance, *O. angasi* displayed an intermediate energy storage strategy between conservative and opportunistic species. The implications of gametogenesis, sex ratio and energy metabolism in reproductive and physiological adaptations of sessile species are discussed.

This chapter has been submitted to Journal of Molluscan Studies as:

Hassan, M. M., Qin, J. G., & Li, X. Reproductive fitness of spermcasting marine bivalve: a case study in the Australian flat oyster *Ostrea angasi*.

Chapter 4 is a review of current research status on oyster sperm cryopreservation. Sperm cryopreservation in broadcasting oysters has progressed substantially but limited information exists on spermcasting oysters. This review chapter explores the lack of standardization in cryopreservation procedures specifically in sperm quality assessment. In addition, sperm concentration remains the most uncontrolled variable for inconsistent outcomes of cryopreservation protocols. The solutions for technical limitations and the scope of application of cryopreserved sperm in oyster aquaculture are explored.

This chapter has been published in Aquaculture as:

Hassan, M. M., Qin, J. G., & Li, X. (2015). Sperm cryopreservation in oysters: a review of its current status and potentials for future application in aquaculture. Aquaculture, 438, 24-32.

Chapter 5 standardizes sperm quantification of *O. angasi* by a spectrophotometric technique (Objective 3). The use of equal amount of sperm is important in a breeding program to optimize sperm to egg ratio and family based genetic improvement programs. Sperm quantification is also important in cryopreservation to optimize cryoprotectant concentrations. In this study, a regression model was developed based on sperm concentration and spectrophotometric absorbance, and sperm concentration was quantified with the regression model. Sperm quantification by the regression model was validated by haemacytometer counts to ensure repeatability of the spectrophotometric technique.

This chapter has been published in Aquaculture Research as:

Hassan, M. M., Qin, J. G., & Li, X. Development of a spectrophotometric technique for sperm quantification in the spermcasting Australian flat oyster *Ostrea angasi* Sowerby. Aquaculture Research. doi: 10.1111/are.13304.

Chapter 6 optimizes the key factors that affect the quality of cryopreserved sperm with the programmable freezing method (Objective 4). Optimization of different factors includes: cryoprotectant toxicity, cryoprotectant types and concentrations, freezing rates, sample volumes and refrigerator storage. Sperm motility and plasma membrane integrity were used as sperm quality assessment indicators. The survival rates of cryopreserved sperm were similar to spermcasting *O. edulis* but relatively lower than other broadcasting species.

This chapter has been published in Cryobiology as:

Hassan, M. M., Li, X., Liu, Y. & Qin, J. G. Sperm cryopreservation in the spermcasting Australian flat oyster *Ostrea angasi* by a programmable freezing method. Cryobiology. doi: 10.1016/j.cryobiol.2017.03.007.

Chapter 7 is a sister chapter of Chapter 6 to improve post-thaw sperm survival (Objective 4). Because freezing with liquid nitrogen vapour produces high survival rate in many marine bivalves, this technique has been optimized by further assessing the key factors that affect post-thaw sperm quality. Optimization of different factors includes: cryoprotectant toxicity, cryoprotectant types and concentrations, distances between sample and liquid nitrogen surface, sample holding durations in liquid nitrogen vapour and sample volumes. Sperm motility and plasma membrane integrity were used as sperm quality assessment indicators. Sperm survival rates between different freezing methods were compared. This study significantly improved the quality of cryopreserved sperm.

This chapter has been submitted to Cryobiology as:

Hassan, M. M., Li, X. & Qin, J. G. Improvement of post-thaw sperm survivals using liquid nitrogen vapour technique in a spermcasting oyster *Ostrea angasi*.

Chapter 8 is a general discussion of the thesis where major research outcomes are integrated. Based on the conclusions of this thesis, further research directions are proposed to tackle those questions beyond the scope of this thesis.

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Chapter 2: Spermatozeugmata structure and dissociation of the Australian flat oyster *Ostera angasi*: implications for reproductive strategy

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2.1 Abstract

Variation in reproductive strategy is one of the key factors contributing to recruitment success of molluscs in different habitats. Spermcasting is a unique mode in mollusc reproduction where males produce spermatozeugmata, a radially arrayed sperm cluster wrapped by gelatinous membrane. In this study, spermatozeugmata structure and their dissociation in the Australian flat oyster *Ostrea angasi* were investigated to elucidate the reproductive strategy in spermacasting molluscs. The histological observation indicated that spermatogonia gradually aggregated in the gonad follicle at the early gonad development stages and developed into spermatozeugmata and became tightly packed at the advanced stages. Even though mature male and female gametes could be found in a hermaphroditic individual, the animal may prevent self-fertilization by shedding different sex gametes at different time. The *O. angasi* sperm are similar in size and shape to broadcasting oysters, but have one additional mitochondrion. Variations in maintaining spermatozeugmata integrity and sperm motility between individuals depended on the level of masculinity or femineity. The durations of spermatozeugmata dissociation and sperm viability were longer in males than in hermaphrodites. The unique structure and capability for spermatozeugmata to maintain the functional integrity after spawning have adaptive significance for fertilization and gamete dispersal in this species.

Keywords: Spermcasting, hermaphrodite, sperm, mollusc

2.2 Introduction

The dynamics of reproductive pattern are vitally important in understanding the life history and adaptation of marine invertebrates. The general mode of reproduction in marine invertebrates is broadcast spawning in which the male and female release

gametes in water where fertilization and embryonic development occur. Spermcasting is another type of reproduction where males broadcast sperm in water and females inhale sperm to fertilize eggs in the body cavity (Bishop and Pemberton, 1997; Pemberton et al., 2003). In most of the spermcasting species, fertilized eggs are incubated inside the female body and develop to a free swimming larva before being released into the water (Jackson, 1985). In this paper, the term 'spermcasting' is adopted from Falese et al. (2011) though its synonyms are also used, such as spermcast mating (Bishop and Pemberton, 2006), egg brooding (Phillippi et al., 2004), egg brooding free-spawner (Johnson and Yund, 2004) and larviparity (Buroker, 1985).

Spermcasting differs from broadcasting in the structure of male gametes. Rather than releasing individual sperm, the spermcasting species spawn sermatozeugmata which can be carried by water movement to the female mantle cavity for fertilization. In a spermatozeugma, the sperm heads are clustered by a gelatinous membrane with tails extending outside (Foighil, 1989). Spermatozeugma is also termed as 'sperm-balls' (Coe, 1931), 'sperm spheres' (Ishibashi et al., 2000) and 'sperm morule' (Jespersen et al., 2001). Spawning of spermatozeugmata is found in some aquatic invertebrates including polychaetes (Drozdov and Galkin, 2012), worms (Maiorova and Adrianov, 2005; Bohn and Heb, 2014), and bivalves (Jespersen et al., 2001; Geraghty et al., 2008), and some vertebrates such as fishes (Meisner et al., 2000; Fishelson et al., 2007).

Past research on sperm structure is mainly focused on broadcasting oysters such as the Pacific oyster *Crassostrea gigas* (Bozzo et al., 1993; Komaru et al., 1994; Dong et al., 2005; Drozdov et al., 2009; Yurchenko, 2012), eastern oyster *C. virginica* (Daniels et al., 1971; Eckelbarger and Davis, 1996), Portuguese oyster *C. angulata* (Sousa and Oliveira, 1994), Iwagaki oyster *C. nippona* (Yurchenko, 2012), Jinjiang oyster *C. rivularis* (Yurchenko, 2012), Sydney rock oyster *Saccostrea commercialis* (Healy and Lester, 1991) and small rock oyster *S. mordax* (Yurchenko, 2012). These

studies have revealed the dimensional differences in sperm components to support species specificity of sperm morphology. As such, the spermatozeugmata dimensions are also different among taxonomic groups (Ferraguti et al., 1989). So far, the oysters that have showed spermcasting behaviour all belong to the genus *Ostrea*; including the European flat oyster *O. edulis* (Foighil, 1989), Chilean oyster *O. chilensis* (Chaparro et al., 1993), Puelche oyster *O. puelchana* (Castanos et al., 2005) and Australian flat oyster *O. angasi* (O'Sullivan, 1980). Among the spermcasting oysters, the spermatozeugma structure is studied only in the European flat oyster while the information on other species is lacking. Furthermore, the implications of spermatozeugmata structures have not been applied to explain the adaptive strategy in reproduction success of spermcasting oysters.

The structural integrity of a spermatozeugma is maintained by gelatinous membrane that envelops sperm heads. However, once the spermatozeugma is released in seawater, sperm become activated and gradually swim-off by dissociating the membrane (Foighil, 1989). A bulk of aggregated sperm in a spermatozeugma improves the fertilization efficiency but only the dissociated sperm can successfully fertilize eggs (Foighil, 1985). In spermcasting oysters, spermatozeugmata could be released by both hermaphroditic and male individuals. In *O. edulis*, spermatozeugma dissociation took place within 24 h after release but the relationship between dissociation rate and the level of masculinity (i.e., testis dominance) or femineity (i.e., ovary dominance) has not been defined. The understanding of the dissociation of spermatozeugmata would provide an insight into the unique reproductive biology of these species.

The Australian flat oyster *O. angasi* is a spermcasting species that release spermatozeugmata to fertilize eggs. Our understanding on the basic biology of *O. angasi* is limited to growth, survival (Dix, 1980; Mitchell et al., 2000), and fertility (O'Sullivan, 1980). After settlement, oysters have no movement capacity to find

potential mates. Therefore, the properties of their gametes are intrinsically related to the evolution of reproductive biology in these species. In this study, we aimed to understand the structure and dissociation of spermatozeugmata in an attempt to elucidate the reproductive strategies of the spermcasting mollusc *O. angasi*.

2.3 Materials and Methods

2.3.1 Oyster source and maintenance

The flat oysters were collected monthly from April to November, 2014 from the Pristine Oyster Farm in Coffin Bay, South Australia and shipped to South Australian Research and Development Institute (SARDI) in a chilled Styrofoam box within 24 h. Our monthly sampling revealed that the oysters with brooded larvae occurred from May to December in South Australia. After arrival, the oysters were cleaned with a brush and kept in a rectangular tank supplied with flow through seawater and aeration. The temperature was maintained at 20 ± 0.5 °C and the oysters were fed with mixed microalgae of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans*. The oysters were two years old having shell length 75.6 ± 5.5 mm and live weight 69.1 ± 13.3 g.

2.3.2 Gonad tissue histology

The gonad of a flat oyster located around the digestive gland and the gonad and non-reproductive tissues are not anatomically separate. The middle portion (3 mm thick) of the gonad-visceral tissues of twenty oysters was cross sectioned each month. Immediately after collection, the gonad-visceral tissues were placed in Davidson's fixative (95% ethyl alcohol - 300 ml, 38% formalin – 200 ml, glacial acetic acid -100 ml and distilled water – 300 ml). The gonad-visceral tissue sections were prepared by an existing histological procedure (Kim et al., 2006). Briefly, the tissues were submerged through the graded alcohol solution and xylene before being embedded in paraffin wax.

A 5 µm cut section was mounted on a microscope slide and stained with haematoxylin and counter stained with eosin. The specimen slides were scanned and the photos were taken on an inverted microscope (Nikon Eclipse TS100-F). The gonad developmental stages in this study were based on the criteria used for the European flat oyster *O. edulis* (da Silva et al., 2009). Briefly, gonad development was categorized into five stages; (i) inactive or resting gonad, (ii) early gametogenesis, (iii) advanced gametogenesis, (iv) mature gonad and (v) spawned gonad. Spermatogonia is undifferentiated germ cells at the early stages of spermatogenesis and spermatocyte is germ cells that arise from spermatogonium. Spermatocytes are more compact and denser than spermatogonia in a developing spermatozeugma. Spermatid is a haploid male gamete that arises from secondary spermatocyte. Spermatids are denser and more compact than spermatocytes in a developing spermatozeugma.

2.3.3 Sperm collection

After the oyster shells were opened, spermatozeugmata/sperm were collected by stripping from the gonad using a 3.5 ml pipette and placed in 1.5 ml Eppendorf tubes. As there were a large percentage of simultaneous hermaphrodites in the flat oyster population, the presence of male gametes in the gonad was confirmed on a light microscope at 200× magnification. The sperm collected from 3-5 individuals were pooled for each motility observation at different masculine levels such as male, predominant male hermaphrodite and predominant female hermaphrodite.

2.3.4 Specimen preparation for electron microscopy

For scanning electron microscope (SEM) observation, the suspended spermatozeugmata in filtered seawater were collected on 0.2 µm polycarbonate membrane filters and placed in a fixative (2.25% glutaraldehyde in phosphate buffer

solution + 4% sucrose, pH 7.2) for 30 min. After fixation, the specimens were placed in a buffer (4% sucrose in phosphate buffer solution) for two consecutive washing of 5 min each. After washing, the samples were post-fixed with 2% osmium tetroxide (OsO_4) for 30 min. The specimens were dehydrated twice in 70%, 90% and 100% ethanol for 10 min each. Then the samples were critical-point dried twice with (i) 1:1 hexamethyldisilazane (HMDS) and 100% ethanol, and (ii) 100% HMDS for 10 min each. The samples were placed in the fume hood at room temperature to vaporize extra moisture. The dried filters were coated with platinum at a thickness of less than 100 Å and six filters were observed with an SEM (Philips XL 30). Sperm from individual male were used and a total of six oysters were used for electron microscopic observations.

2.3.5 Dissociation of spermatozeugmata

Sperm surrounded by a gelatinous membrane were considered non-dissociated whereas sperm swimming freely were considered dissociated. The dissociation of spermatozeugmata was observed by placing an aliquot of 20 µl suspension on a glass slide under a light microscope at 200× magnification. The time required for the dissociation of spermatozeugmata collected from the male, predominant male hermaphrodite and predominant female hermaphrodite was compared.

2.3.6 Sperm motility

Although the sperm attached to a spermatozeugma had flagella beating but this effect was not considered while studying sperm motility because the number of sperm with an active flagellum in a spermatozeugma cannot be quantified. The typical individual sperm with active forward movement was counted motile while those without such a movement were considered non-motile. The sperm suspension was produced by passing the sample through a 45 µm mesh. The 45 µm nylon mesh was

fitted between two 1 mL pipette tips and filtration was achieved by creating a downward force with the pipette. The inner tip was cut by approximately 3 cm from narrow end. A 2 µl sperm suspension was placed on a glass slide and 20 µl filtered seawater was added to activate the sperm and motility was observed at 20^{o} C. In this study, the duration of sperm motility was defined as the period from the time when sperm were collected to when all sperm in a randomly selected field stopped forward movement at a standardised sperm concentration. The duration of motility of sperm collected from the male, predominant male hermaphrodite and predominant female hermaphrodite was compared.

2.3.7 Statistical analysis

The duration of spermatozeugma dissociation or sperm motility among the male, predominant male hermaphrodite and predominant female hermaphrodite was compared using one-way analysis of variance (ANOVA). The least-significant difference (LSD) comparison was used while the differences considered statistically significant at $P < 0.05$. The results were presented as mean ± SD. Data were analysed by SPSS version 20.0 (IBM Corporation, Armonk, NY, USA).

2.4 Results

2.4.1 Spermatozeugmata formation during spermatogenesis

While the gonad was at a resting stage during spermatogenesis, only a few spermatogonia were present in the follicle (Fig. 1a). Spermatogonia are relatively large, prominent and bulky bundles, and mainly present in the early stage of spermatogenesis (Fig. 1b). The encapsulated sperm bundles were packed in the follicles at the advanced stage of spermatogenesis and became smaller, more compact and darker to form a spermatocyte (Fig. 1c, d). There were free spermatogonia in the gonad epithelium at the

early stage but no free spermatid was found at the advanced stage. Although a spermatogonium was comparatively large in diameter it became small at the spermatocyte stage. When the flat oyster was close to spawning, sperm in a fully matured spermatozeugma were much dense and compact. Within an individual oyster, the degrees of spermatozeugmata maturity were different (Fig. 1d), indicating the likelihood of spawning in multiple batches.

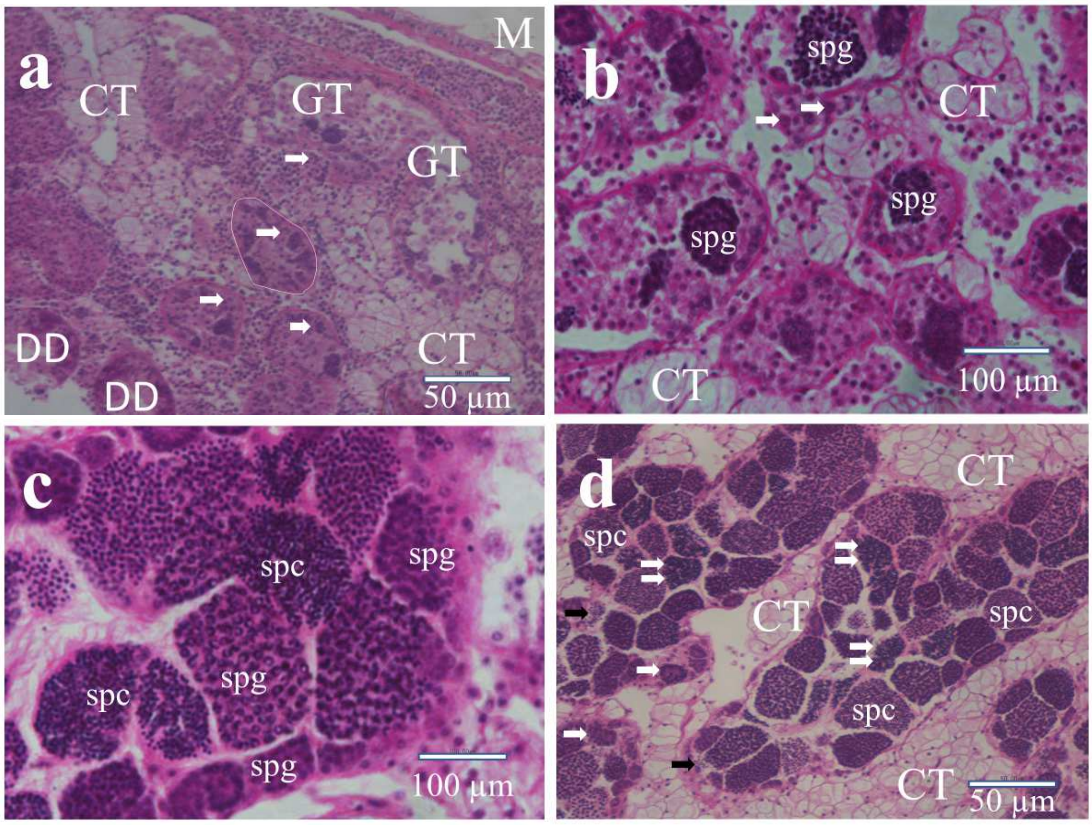

Fig. 1: Histological sections of *Ostrea angasi* showing the formation of spermatozeugmata during spermatogenesis in males. Each bundle in the gonad follicle represents a developing spermatozeugma. a) inactive or resting gonad: almost empty follicles between mantle and digestive gland, rudimentary spermatozeugmata (arrows) and gonad follicles (ellipse circle); b) early stages of spermatogenesis: enlarged follicles containing spermatogonia clusters and free spermatogonium (arrows); c) advanced spermatogenesis: follicles filled with spermatogonia and spermatocyte, and d) mature

gonad: compact and dense follicles containing spermatocyte and spermatid, spermatogonia (white arrow), spermatid (double arrows) and phagocytes (black arrow). CT: connective tissues; DD: digestive diverticula; GT: gonad tubules; M: mantle; spc: spermatocyte, and spg: spermatogonia.

The flat oyster had a unique reproductive system representing three sex categories: male, female and hermaphrodite. Based on the relative proportion of male and female gametes, the hermaphroditic gonads were further classified into five levels; (i) hermaphrodite with predominant male gametes: follicles filled with approximately 60-90% of male gametes and 10-40% female gametes, (ii) hermaphrodite with predominant female gametes: follicles filled with approximately 60-90% of female gametes and 10-40% male gametes, (iii) hermaphrodite with rudimentary female gametes: follicles filled with <10% of female gametes and >90% of male gametes, (iv) hermaphrodite with rudimentary male gametes: follicles filled with <10% male gametes and >90% of female gametes, and (v) hermaphrodite representing a similar proportion of both gametes (Fig. 2). There was also a pattern in the relative position of male and female gametes in the hermaphrodite gonad follicle in which male gametes are generally surrounded by a layer of female gametes.

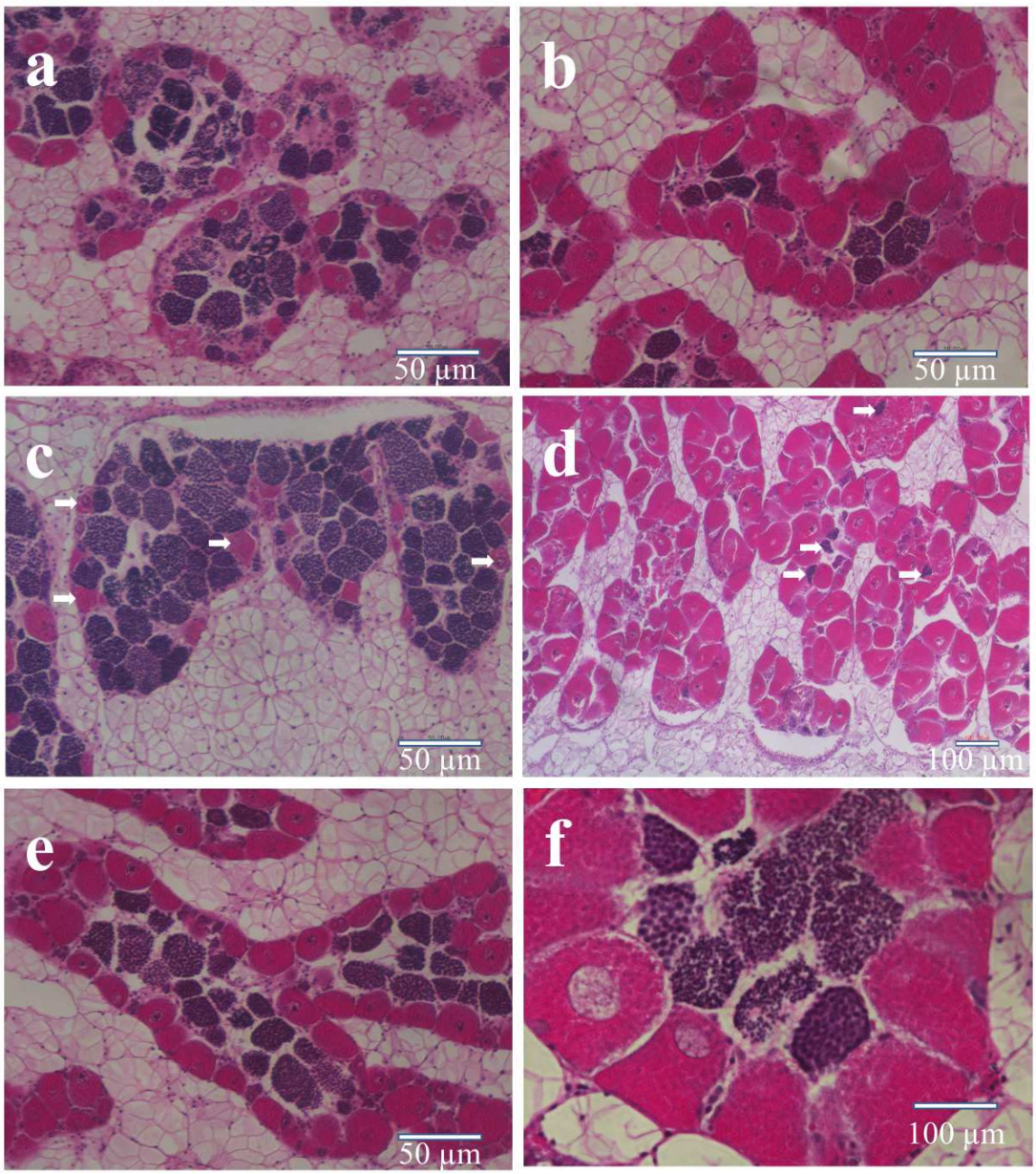

Fig. 2: Presence of spermatozeugmata in hermaphrodite *Ostrea angasi*; a) hermaphrodite with predominant male gametes, b) hermaphrodite with predominant female gametes, c) hermaphrodite only with rudimentary oocytes (arrow), d) hermaphrodite only with rudimentary spermatocytes (arrow), and e and f) both sex gametes nearly equal.

2.4.2 Spermatozeugmata morphology

In a spermatozeugma, sperm heads were bounded in a well-defined gelatinous membrane, whereas the tails were projected outside (Fig. 3a). The gelatinous membrane

was worn out during the sample fixation for electronic microscopy (Fig. 3b). The mean diameter of spermatozeugmata was 116.8 ± 38.4 µm (n = 6).

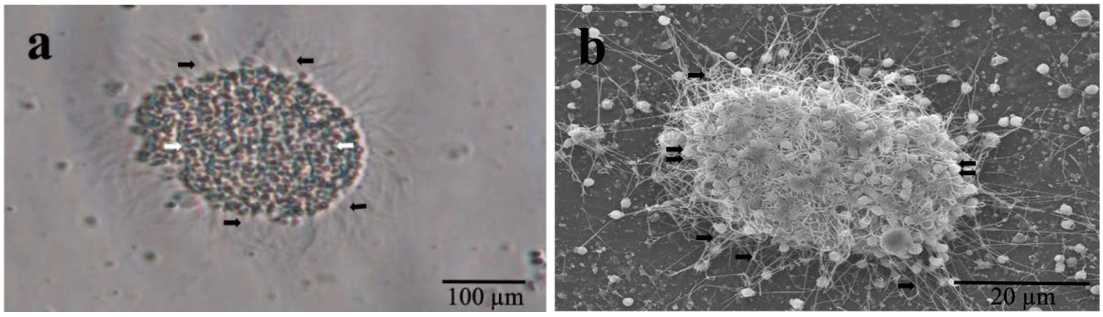

Fig. 3: Contrasting views of a spermatozeugama of *Ostrea angasi*; a) light microscope image: sperm head (white arrow) and tail (black arrow); and b) scanning electron microscopic image: sperm head (double arrows) and tail (single arrow).

2.4.3 Sperm morphology and integrity during fixation

Although the fertilization process in flat oysters was different from most other marine animals, the sperm structure of flat oyster was considered 'primitive' (Franzén, 1970) because the sperm had a radially symmetrical cell body with a bullet-shaped head and a long flagellum (Fig. 4a). The flat oyster sperm had five mitochondria to provide the energy for flagellum beating during movement (Fig. 4b). The diameter of a mitochondrion was 0.4 ± 0.0 µm (n = 10). The sperm head length and width were 1.9 ± 0.1 µm (n = 12) and 1.6 ± 0.1 µm (n = 12), respectively, and the ratio of head length to width was 1.2. A variation in the sensitivity of sperm to the fixation method was found while preparing specimen for electron microscope imaging. The same fixation method rendered unimpaired sperm structure (Fig. 4a). However, it could also cause membrane disruption, tail detachment and indentation in the mitochondrial region (Fig. 4b, 5a and 5b).

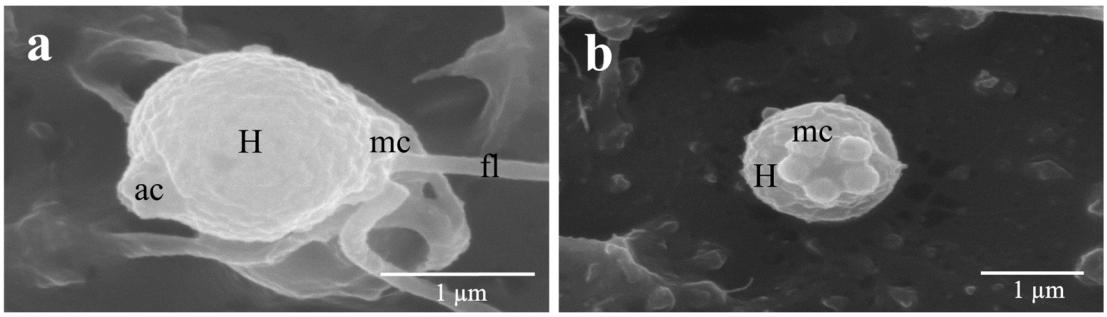

Fig. 4: Scanning electron micrograph of *Ostrea angasi* sperm; a) sperm head, b) five mitochondria exposed after membrane disruption. ac: acrosome, fl: flagellum, H: head and mc: mitochondria.

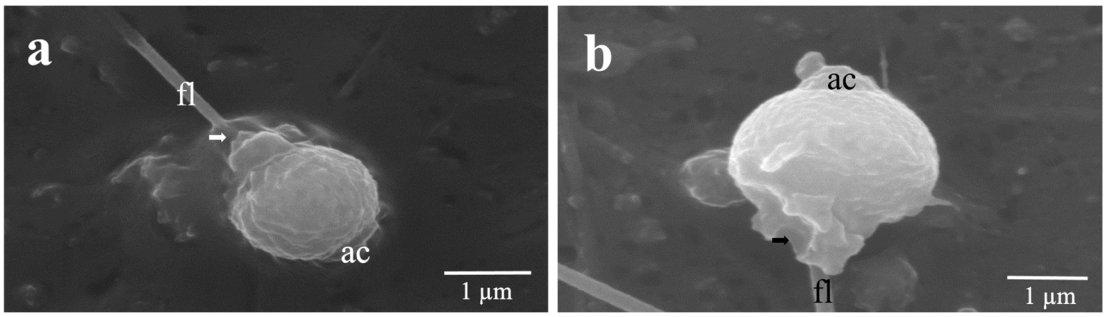

Fig. 5: Damage of *Ostrea angasi* sperm during fixation for electron microscopy; a) detached tail, and b) indentation in the mitochondrial region. ac: acrosome and fl: flagellum.

2.4.4 Spermatozeugmata dissociation

During dissociation in seawater, the extracellular matrix of a spermatozeugma was disintegrated, leaving the isolated patches of the sperm relics. The concentration of dissociated sperm increased with the increase in activation duration. The duration of spermatozeugma dissociation of male, predominantly male hermaphrodite and predominantly female hermaphrodite was 19.7 ± 2.8, 10.2 ± 1.9 and 3.5 ± 1.6 h, respectively. There were significant differences in the spermatozeugma dissociation duration among the three masculine levels ($F_{2, 42} = 139.97$, $P < 0.001$) (Fig. 6).

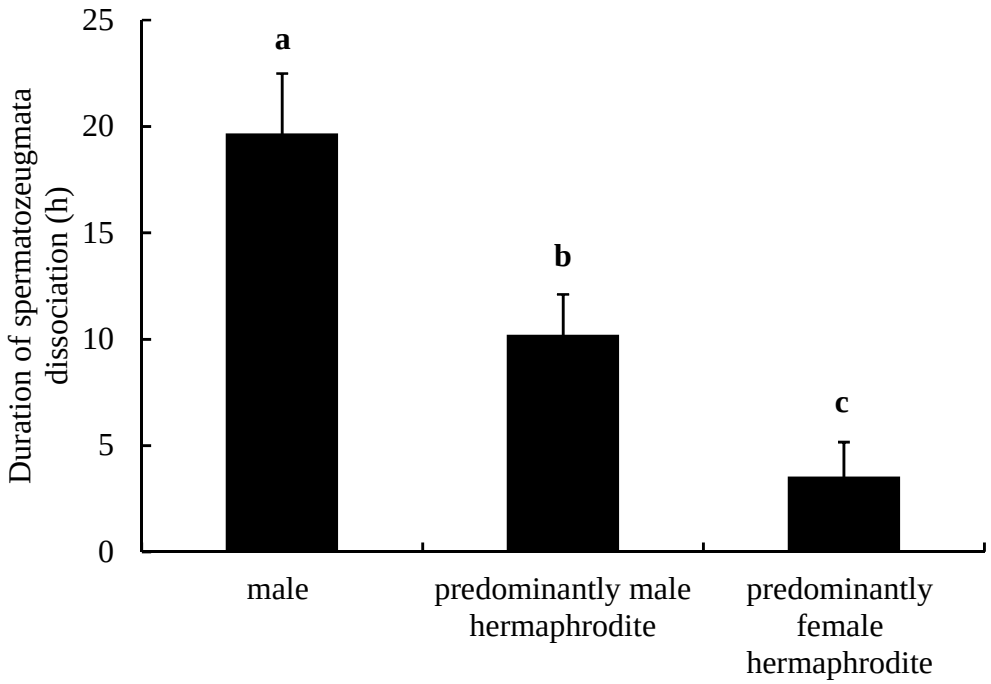

Fig. 6: The duration of spermatozeugma dissociation of three masculine levels in *Ostrea angasi* exposed to filtered seawater. Different letters indicate significant differences among the masculine levels. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SD of five replicates.

2.4.5 Sperm motility

Sperm started swimming immediately after activation in broadcast spawners, whereas sperm motility in flat oysters had two phases based on the tempo and magnitude of flagella beating action: (1) low flagella beating while attached to a spermatozeugmata and (2) high flagella beating after the break-off from spermatozeugmata membrane. Sperm collected from males maintained longer motility duration compared to hermaphrodites. The duration of sperm motility of male, predominantly male hermaphrodite and predominantly female hermaphrodite was 20.1 ± 2.8, 10.8 ± 2.0 and 4.8 ± 1.7 hours, respectively. There were also significant differences in the duration that a sperm maintained swimming capability among the three masculine levels ($F_{2, 42} = 166.04$, $P < 0.001$) (Fig. 7).

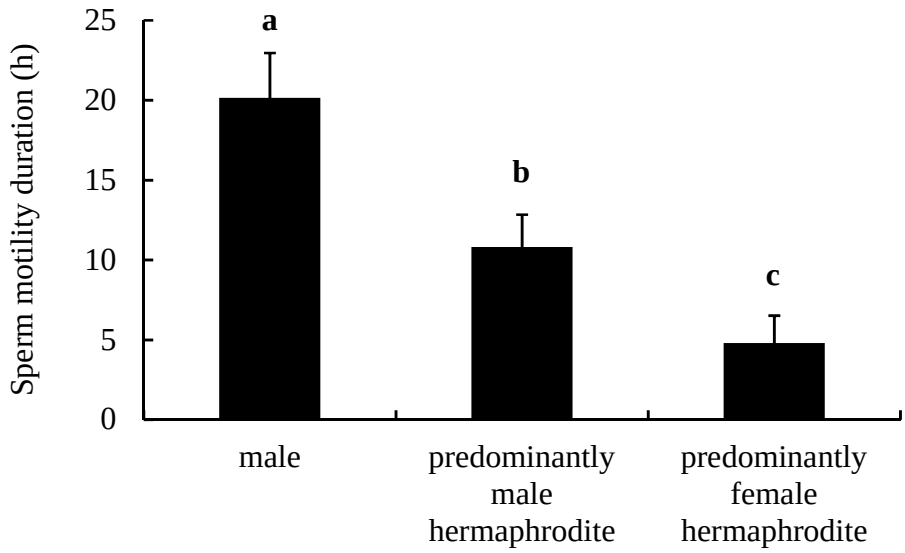

Fig. 7: Motility duration of sperm of three masculine levels of *Ostrea angasi* exposed to filtered seawater. Different letters indicate significant differences among the masculine levels. Sperm from three to five males were pooled in a replicate. Each bar represents the mean ± SD of five replicates.

2.5 Discussion

This study revealed the pattern of spermatozeugma development in the Australian flat oyster *O. angasi* during spermatogenesis. The formation of spermatozeugmata is discussed based on the proliferation and aggregation of germ cells in the gonad follicles. At the beginning of spermatogenesis, the follicles were partly filled with the primary spermatozeugmata as a cluster of spermatogonia (<100 sperm). At this stage, both clusters of spermatogonia and free spermatogonium were present in the gonad. With the progress of spermatogenesis, spermatogonium started to condense but the area occupied by gametes in the follicles gradually increased. At the advanced stage of spermatogenesis, the individual gametes were compacted and no free spermatid was found in the gonad follicles. A pattern of male gamete condensation due to shrinkage in the diameter of sperm nucleus at the early stages of development was also

observed in the Pacific oyster (Franco et al., 2008). The concurrence of spermatogonia, spermatocyte and spermatid in the gonad of an individual suggests that a multiple spawning strategy may exist in *O. angasi*. The multiple spawning of this species as hypothesized from the micrograph of gonad histology is also supported by the microscopic observation of spent individuals. The stripped spawning of spent individuals revealed relict of mature spermatozeugmata in the gonad.

Oysters in the genus *Ostrea* are protandric hermaphrodite, i.e., the individuals mature as a male and change the sex from a male to female in life history (Orton, 1933). Simultaneous hermaphroditism is also common in *O. edulis* (Wilson and Simons, 1985; da Silva et al., 2009), *O. chilensis* (Jeffs, 1998) and *O. lurida* (Coe, 1932). However, the term 'simultaneous hermaphroditism' does not explain the magnitude of different sex gametes in the gonad because hermaphrodites may have varying proportions of male and female gametes between individuals. The magnitude of masculinity or femineity in a hermaphrodite *O. angasi* ranged from highly skewed to one sex to almost equal in both sexes. Such hermaphroditism, either skewed to one sex or equal proportion of both sexes was also found in the European flat oyster (da Silva et al., 2009). In Australian flat oysters the dynamic of protandry has not been investigated. Literature in other oysters suggests that the proportion of hermaphrodite individuals in a population is influenced by environmental factors such as temperature and photoperiod (Joyce et al., 2013), food availability (González-Araya et al., 2013) and hereditary constituents (Guo et al., 1998; Harding et al., 2013).

The mechanism to avoid self-fertilization in simultaneous hermaphrodite individuals has never been clarified in the flat oyster species. Self-fertilization refers to the fusion of male and female gametes from the same individual to produce a zygote, and this phenomenon also occurs in some gastropods (Chen, 1994; Jarne et al., 1991), and other bivalves (Kurihara et al., 2010). Based on the presence of mature eggs and

spermatozeugmata in the same individual, our study invokes two fundamental questions: (1) is self-fertilization a reality in flat oysters? and (ii) if not, how do flat oysters avoid self-fertilization in nature? Yet, there has been no evidence of self-fertilization in Ostreidae and this could be achieved by releasing the matured male and female gametes of an individual at different time intervals that the self-fertilization can be avoided. However, in the hermaphrodite tunicate *Pyura chilensis*, self-fertilization occurs as an alternative mode of reproduction when the tunicates are captivated in solitary (Manríquez and Castilla, 2005). Further investigations are needed to reveal if similar phenomenon can occur in the simultaneous hermaphrodite flat oysters.

The evolution of gamete structure in different species may follow the physiological demand of their fertilization process. The production of spermatozeugmata is one of the mechanisms for indirect sperm transfer in sessile brooders. As the number of sperm in a cluster is large, the intake of a few clusters would have enough sperm to effectively fertilize the eggs in a female. The flat oysters become sessile after metamorphosis resulting in spatial constraints in gamete dispersals. Unless transported by flagella beating and water current, the male gamete cannot reach females (Le Pennec and Beninger, 2000). Therefore, the intake of spermatozeugmata would facilitate the fertilization in the mantle cavity of spermcasting species. The morphological features of spermatozeugmata in spermcasting oysters are similar, and the sperm head is bounded by a gelatinous membrane and the tail extends outside. However, the mean diameter of a spermatozeugmata was 117 µm in *O. angasi* which is larger than that of *O. edulis* (Foighil, 1989).

Oyster sperm have common morphological features among species. The overall structure of *O. angasi* sperm is similar to other species in the family Ostreidae in acrosome shape, sperm head, mitochondrial region and flagellum. Oyster sperm also have species specific linear dimensions and length to width ratios (Yurchenko, 2012).

The sperm head length and width are respectively 2.6 and 2.3 µm in *C. gigas* (Dong et al., 2005), 1.4- 1.9 and 0.8-1.4 µm in *C. virginica* (Galtsoff and Philpott, 1960) and 1.9 and 1.6 µm in *O. angasi* (current study). The ratio of sperm head length to width is 1.1 in *C. gigas* (Dong et al., 2005) and 1.2 in *O. angasi* (current study). Overall, the bullet-shaped sperm head and a long tail are an adaptation of sperm morphology to swim a longer distance (Suquet et al., 2012).

The number of mitochondria in sperm is an interesting topic because mitochondria play an important role in sperm motility by supplying energy for movement. We found five mitochondria in *O. angasi*, whereas all broadcasting diploid oysters investigated so far only have four mitochondria (Yurchenko, 2012). The tetraploid Pacific oysters, on the other hand, have four to six mitochondria (Dong et al., 2005). This trend of increase in the mitochondrial number with the increase in ploidy level also occurs in Mediterranean mussels *Mytilus galloprovinciallis*, with diploid and tetraploid counterparts having five and five to seven mitochondria, respectively (Komaru et al., 1995). The intra-specific comparison on the number of mitochondria among different levels of ploidy reveals that the individuals with higher ploidy levels also have a bigger sperm head, which normally contains more mitochondria. But sperm with a larger head contains more mitochondria may not always be true for inter-specific comparisons. The reason for the additional number of mitochondria in tetraploids is probably due to more energy requirement to drive a larger sperm head in tetraploid bivalves. In the sperm of other bivalve species, the number and diameter of mitochondria are species specific (Table 1).

Table 1: Sperm head dimension, mitochondria number and diameter in some bivalves

Species	Sperm head length × width (µm)	Mitochondria number	Mitochondria diameter (µm)	References

Arca boucardi	3.2 × 2.6	5	0.8	Drozdov et al., 2009
Crassostrea gigas (diploid)	2.6 × 2.3	4	-	Dong et al., 2005
	2 × 2.5	4	0.8	Drozdov et al., 2009
C. gigas (tetraploid)	3.4 × 3.0	4-6	-	Dong et al., 2005
Mya japonica	5.5 × 1.5	4	0.8	Drozdov et al., 2009
Mytilus galloprovincialis (diploid)	-	5	-	Komaru et al., 1995
M. galloprovincialis (tetraploid)	-	5-7	-	Komaru et al., 1995
Ostrea angasi	1.9 × 1.6	5	0.4	Present study
Pododesmus macrochisma	5.2 × 2	4	1	Drozdov et al., 2009
Trapezium liratum	2.6 × 1.4	5	0.6	Drozdov et al., 2009

In the present study, many sperm observed on a scanning electron microscope had a broken tail, ruptured membrane and dented head. These morphological anomalies have also been observed in *C. gigas* sperm fixed in the non-isotonic solution (Dong et al., 2006). In this study although the sperm were initially suspended in isotonic

seawater, the osmolality was unknown during sperm fixation in glutaraldehyde and post-fixation in osmium tetroxide. It is possible that the damages were caused by one of the following factors or their combination: (1) there is a difference in sensitivity to the fixation procedure between sperm at different maturity, and (2) the *O. angasi* oyster sperm are more sensitive to the fixation method used in this study than on other species. Future research is needed to investigate if other fixation methods are more suitable for scanning electron microscope observations in bivalves.

The time when sperm swim off a spermatozeugma varied significantly between individuals at different masculine levels in *O. angasi*. The mean duration of spermatozeugma dissociation was 19.7 h in 'pure' males, whereas it depended on the proportion of male and female gametes in the gonad of hermaphrodite individuals; the higher the proportion of female gametes, the quicker the dissociation of spermatozeugmata in seawater. This was the first study that unveiled the magnitude of spermatozeugma dissociation relating to masculinity or femineity in aquatic species. Further study is needed to investigate its implication in the reproduction strategy in this species.

Unlike the teleost sperm where motility lasts for only a few seconds to minutes, oyster sperm in general have motility duration from hours to days. The average motility duration of *O. angasi* sperm was 20.1 h (present study) whereas the Pacific oyster sperm had a movement duration of up to 24 h (Suquet et al., 2012). Sperm of spermcasting oysters have two phases of motility (being attached to a spermatozeugma and free swimming), whereas those of the broadcasting oysters only have the free swimming phase. Like spermatozeugma dissociation, there were variations in the sperm motility duration among males and hermaphrodites. The sperm collected from the 'pure' males maintained a longer motility duration than the sperm of hermaphrodites. This result leads us to hypothesize that although sperm of the hermaphrodites are

capable to take part in the fertilization process but they might be less competitive than a 'pure' male if brooders locate in a far distance as sperm of hermaphroditic individuals have a shorter life. However, when brooders are in the proximity, the duration of sperm integrity or motility would have less effect on the fertilization success. In another spermcasing oyster *O. puelchana*, this potential distance impact has been overcome by directly releasing male gametes into the mantle cavity of the female where the dwarf male attached (Pascual, 1997).

In conclusion, this study examined the development of spermatozeugmata, sperm morphology, and the post-spawning dissociation of spermatozeugmata in the Australian flat oyster *O. angasi*. The understanding of these processes would provide basic information to further unveil the mechanisms controlling spermatozeugmata acquisition and storage in the female mantle cavity, and the reproductive strategy in spermcasting species.

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Chapter 3: Gametogenesis, sex ratio and energy metabolism in the spermcasting Australian flat oyster *Ostrea angasi*: implications for reproductive and physiological adaptation

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3.1 Abstract

Spermcast spawning is a unique reproductive strategy of some sessile marine invertebrates such as the oysters in the genus *Ostrea* in which a functional male releases spermatozeugmata to fertilize eggs inside the body cavity of a functional female. Oysters of the genus *Ostrea* have high ecological and economical importance but their adaptive strategies in spermcast spawning remain poorly understood. This study elucidates how gametogenesis, sex ratio and energy metabolism regulate the reproductive and physiological fitness in spermcast spawner through monthly sampling of a representative species *O. angasi* over a year. Gonad histology indicated that spermatozeugmata developed asynchronously within an individual but the oocytes matured synchronously. The hermaphroditic individuals spawned spermatozeugmata before egg ovulation. The population of 2-3 years old oysters comprised 46.7% hermaphrodites and displayed a highly-skewed male to female ratio of 7:1. This species primarily metabolized glycogen as energy source for gametogenesis, with the periods of energy storage and energy utilization being overlapped. The gametogenesis pattern suggests multiple productions of spermatozeugmata in a reproductive season in male and hermaphroditic oysters but single episode of egg ovulation in female and hermaphroditic oysters. The high proportion of hermaphrodites and skewed male to female ratio are generally common among spermcasting species when they are young or smaller in sizes. The energy metabolism indicates that *O. angasi* follows an intermediate strategy between conservative and opportunistic species. The gamete development pattern, skewed sex ratio and energy metabolism strategy are of significance to reproductive and physiological adaptations in spermcasting molluscan species.

Keywords: Reproductive strategy, spermatozeugmata, fertilization, spawning, mollusc

3.2 Introduction

The recruitment success of a marine invertebrate depends the development of an appropriate reproductive strategy(ies) during the life history. Diverse reproductive strategies have evolved in different marine invertebrates to maximize species fitness in the context of reproductive success. Broadcast spawning is a mode of reproduction strategies commonly shared in most marine invertebrates while other species such as some molluscs have developed a unique strategy - spermcasting spawning to provide maternal protection for embryos and larvae in the mantle cavity (Bishop and Pemberton, 2006; Mardones-Toledo et al., 2015). To increase the reproductive success, different reproductive systems such as gonochorism, sequential and simultaneous hermaphroditism have evolved in different species. Strategies for allocating more energy to reproduction are fundamental trade-offs to maximize species fitness in the life history (Llodra, 2002). Energy is obtained from either recently ingested food in opportunistic species or pre-stored in conservative species prior to gametogenesis (Bayne, 1976).

Although oysters of the family Ostreidae are generally considered protandric hermaphrodites, i.e., a male changes sex to a female at a certain stage of the life cycle but sex changes in both directions are also observed in eastern oysters *Crassostrea virginica* (Yang et al., 2015). The mode of spawning of the oysters differs among genera. Males/ hermaphrodites in the genus *Ostrea* release sperm as spermatozeugma (also called spermcast spawning) that are inhaled into the female mantle cavity to fertilize the newly released eggs. The subsequent larval development is protected by the female inside this cavity (Chaparro et al., 2006). In the genus *Crassostrea*, on the other hand, both types of gametes are released into and resultant embryos develop in the water column (broadcast spawning). Although oysters of the genus *Ostrea* are of

immense ecological and fishery importance, the adaptive strategies in spermcast spawning remain poorly understood.

Gametogenesis process is useful to elucidate the reproductive strategy of a species. A synchrony in maturation of the male and female gametes during gametogenesis leads to synchronous spawning, whereas discrepancy in gamete development leads to asynchronous spawning (Styan and Butler, 2003; Chaves-Fonnegra et al., 2016). Since spermcast spawners are incapable of movement after settlement, acquisition of functional spermatozeugmata by a female is necessary for successful reproduction. The development patterns of spermatozeugmata and eggs provide a cue to understand the spawning biology of spermcasting species (Foighil, 1989; Falese et al., 2011). Depending on the reproductive strategy of a species and environmental cues, gametogenesis is either restricted to a certain period or protracted throughout the year. As such, gametogenesis can explain the temporal pattern of reproduction of a species.

Sex change is a regulatory strategy to optimize reproductive output. The 1:1 male to female ratio is common in gonochoristic species (Fisher, 1930), but when reproductive output of hermaphrodite species exceeds the capacity to support progeny survival by the available resources, animals can switch to an opposite sex to adjust the quantity of reproductive output to improve species fitness (Charnov, 1982). Although all the species in the family Ostreidae are protandric hermaphrodites, the sex ratio varies among the broadcasting and spermcasting species (Steele and Mulcahy, 1999; Vaschenko et al., 2013; Acarli et al., 2015). This variation makes species in the Ostreidae family a suitable model to study sex ratio in relation to spawning strategy. The sex ratio needs to be examined before the recruitment pattern of a protandric species can be determined in the family Ostreidae.

The energy metabolism in marine invertebrates is closely associated with reproductive activities. Energy is stored as glycogen, protein and lipid in various tissues and mobilized for physiological and reproductive activities (Berthelin et al., 2000; Benomar et al., 2010). The dynamics in biochemical compositions in the storage tissues and body condition index reveal the energy metabolism strategy of a species. As gametogenesis is a high energy demanding process, marine bivalves use either opportunistic or conservative strategy to control energy expenditure during gametogenesis (Bayne, 1976). In a temperate region, temperature and food are seasonally variable and environmental variation regulates the reproductive clock (Garrido and Barber, 2001; Enríquez-Díaz et al., 2009) that results in most species to follow the conservative energy metabolism strategy (Darriba et al., 2005; Li et al., 2006; Karray et al., 2015). However, the energy metabolism strategy needs to be evaluated for a species which has a protracted spawning period and grows in an area with low food supply such as *O. angasi* to understand how energy is fuelled for a long spawning season without much calorie intake.

The endemic flat oyster *O. angasi* used to be abundant in Australia, but was almost perished from the natural habitat in the late 18th and early 19th century due to overfishing (Nell, 2001; Alleway and Connell, 2015). Since the last decade, the production of *O. angasi* has increased and it has become an emerging species for aquaculture in Australia (Heasman et al., 2004; O'connor and Dove, 2009). In recent years, flat oyster reef restorations have started to repair the bays and estuaries that locals rely on. Despite the importance of this species to fishery, aquaculture and ecological system, some fundamental questions pertaining to its unique spawning strategy remain unanswered. The objectives of this study are to elucidate the adaptive strategies of spermcasting species by answering the following questions: (1) How does

gametogenesis facilitate spermcast spawning? (2) How does sex ratio regulate spawning success in a spermcasting population? and (3) Which metabolism strategy does spermcasting species adopt to allocate energy for reproduction?

3.3 Materials and methods

3.3.1 Oyster and seawater sampling

Monthly collection of seventy flat oysters and seawater was conducted at the leases of Pristine Oyster Farm in Coffin Bay, South Australia from April 2014 to May 2015. Oysters were transported within 24 h in a chilled Styrofoam box to the laboratory at Flinders University. The oysters were then cleaned with a brush and blotted using paper towel prior to length and weight measurements. Oysters were assigned randomly into three groups to study gonad histology, biochemical composition and condition index. The age of oysters in this study was 2-3 years old (76.8 ± 5.2 mm shell length and 72 ± 10.9 g wet weight).

3.3.2 Environmental parameters

Monthly measurement of environmental variables included water temperature, salinity and chlorophyll *a*. After filtering out debris and zooplankton through a 300 μm screen, I collected the phytoplankton on a 0.45 μm Millipore filter, wrapped the Millipore filter with aluminium foil, and stored at –20 ^{o}C for <7 days before analysis. Chlorophyll *a* was extracted by dissolving the filter paper in 90% acetone and stored at 4 ^{o}C for 24 hours. The determination of chlorophyll *a* concentration was on a microplate reader (CLARIOstar, BMG Labtech) at the wavelengths of 647 nm and 664 nm, using the formula: Chlorophyll *a* (mg L^{-1}) = 11.87_{A664} -1.786_{A647} for calculations (Ritchie, 2006), where A664 and A647 represent absorbance at 664 nm 647 nm, respectively.

3.3.3 Histological observation on gonad tissues

The procedures for histological preparation of *O. angasi* gonad-visceral tissues followed an early paper by Hassan et al. (2016). In brief, the protocol included fixing 3 mm cross sectioned middle portion of gonad-visceral tissues in Davidson's fixative (95% ethyl alcohol - 300 ml, 38% formalin – 200 ml, glacial acetic acid -100 ml and distilled water – 300 ml), submerging the specimens in different grades of alcohol and xylene, embedding them in paraffin wax, cutting a 5 µm cross section, mounting the section on a microscope slide, staining with haematoxylin, and counter-staining with eosin. The histological specimen slides were photographed on an inverted microscope (Nikon Eclipse TS100-F).

The gonad development of this species was described according to the following five stages (Fig. 1 and 2):

Inactive or resting gonad (stage 1): Gonads of sexually immature and recently-spawned individuals. Immature gonads comprise gonadal ducts but post-spawned gonads comprise inflated empty follicles. Connective tissues predominantly filled the spaces between the mantle and digestive gland. Presence of rudimental spermatogonia and up to 30-µm diameter oocytes.

Early gametogenesis (stage 2): Gonad follicles are larger and occupied with more gametes than in the previous stage. Gonad follicles and connective tissues almost equally share the space between mantle and digestive gland. Presence of small spermatogonia clusters and 30-60-µm diameter developing oocytes.

Advanced gametogenesis (stage 3): Gonad follicles are larger than the previous stage and occupy over fifty percent of the areas between the mantle and the digestive gland. The gametes occupy majority of the area in the gonad follicles. Oocytes are 60-80-µm in diameter.

Ripe gonad (stage 4): Large gonad follicles occupy the entire area between the mantle and the digestive gland. Mature oocytes of >80-µm diameter are present.

Spawned gonad (stage 5): Gonad follicles are partially or almost completely empty. Presence of phagocytes and residual gametes in the gonad follicles, and larvae in the mantle cavity.

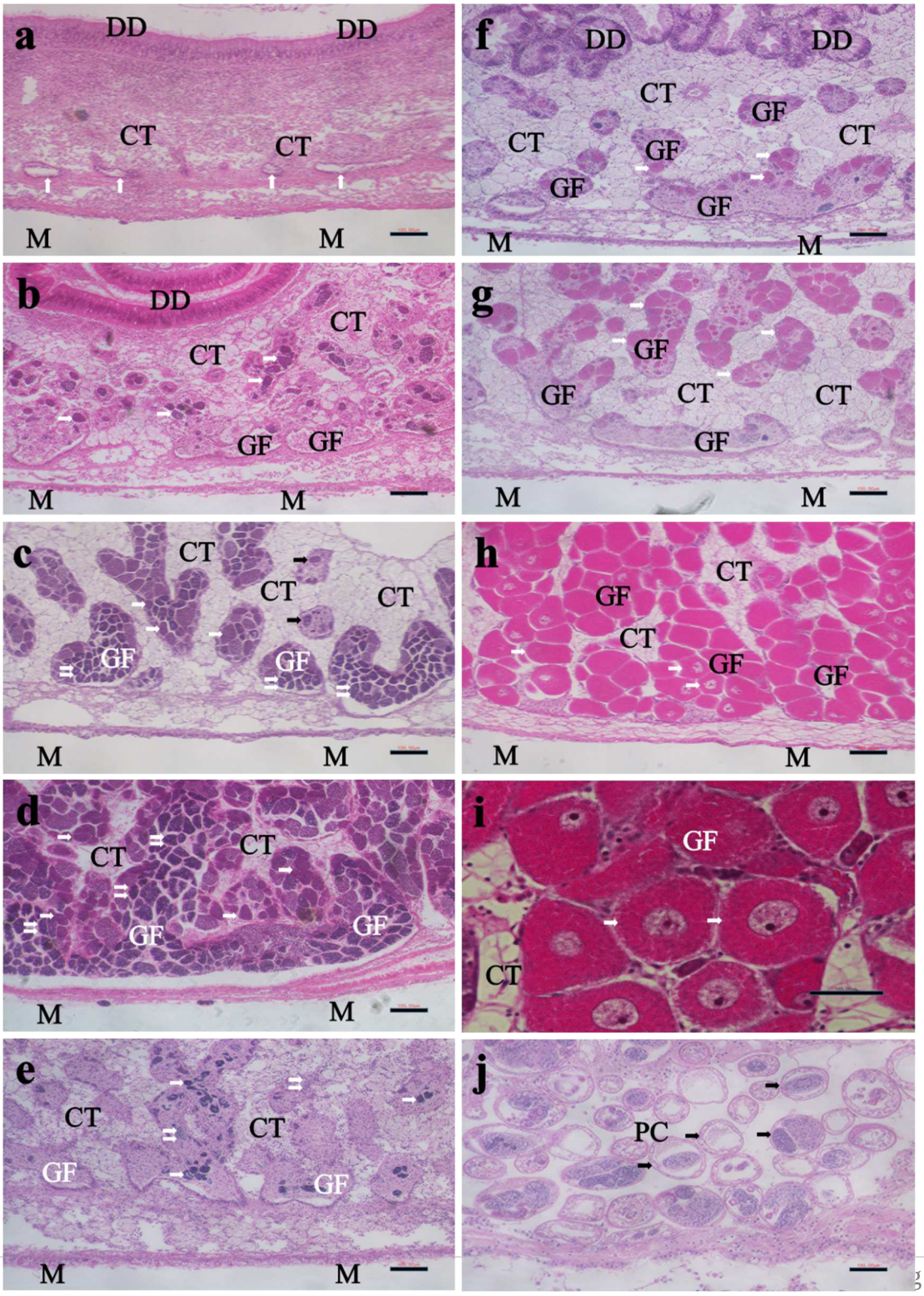

Fig. 1: Histological sections of male (left) and female (right) *Ostrea angasi* at different stages of gonad development. a) male, inactive or resting gonad. Arrows indicate developing gonad follicles between mantle and digestive gland. b) male, early spermatogenesis. Arrows indicate developing spermatogonia in the gonad follicle. c) male, advanced spermatogenesis. Black, single white and double white arrows indicate spermatogonia, spermatocytes and spermatids, respectively. d) male, ripe gonad. Single and double arrows indicate spermatocytes and spermatids, respectively. e) male, partially spawned gonad. Single and double arrows indicate residual spermatids and phagocytes, respectively. f) female, inactive or resting gonad. Arrows indicate rudimentary oocytes in the gonad follicle. g) female, early oogenesis. Arrows indicate developing oocytes in the gonad follicle. h) female, advanced oogenesis. Arrows indicate medium sized oocytes. i) female, ripe gonad. Arrows indicate mature oocytes. j) female, presence of larvae (arrow) in the pallial cavity in spawned oyster. CT: connective tissue; DD: digestive diverticula; GF: gonad follicle; M: mantle; PC: pallial cavity. Bar = 100 µm.

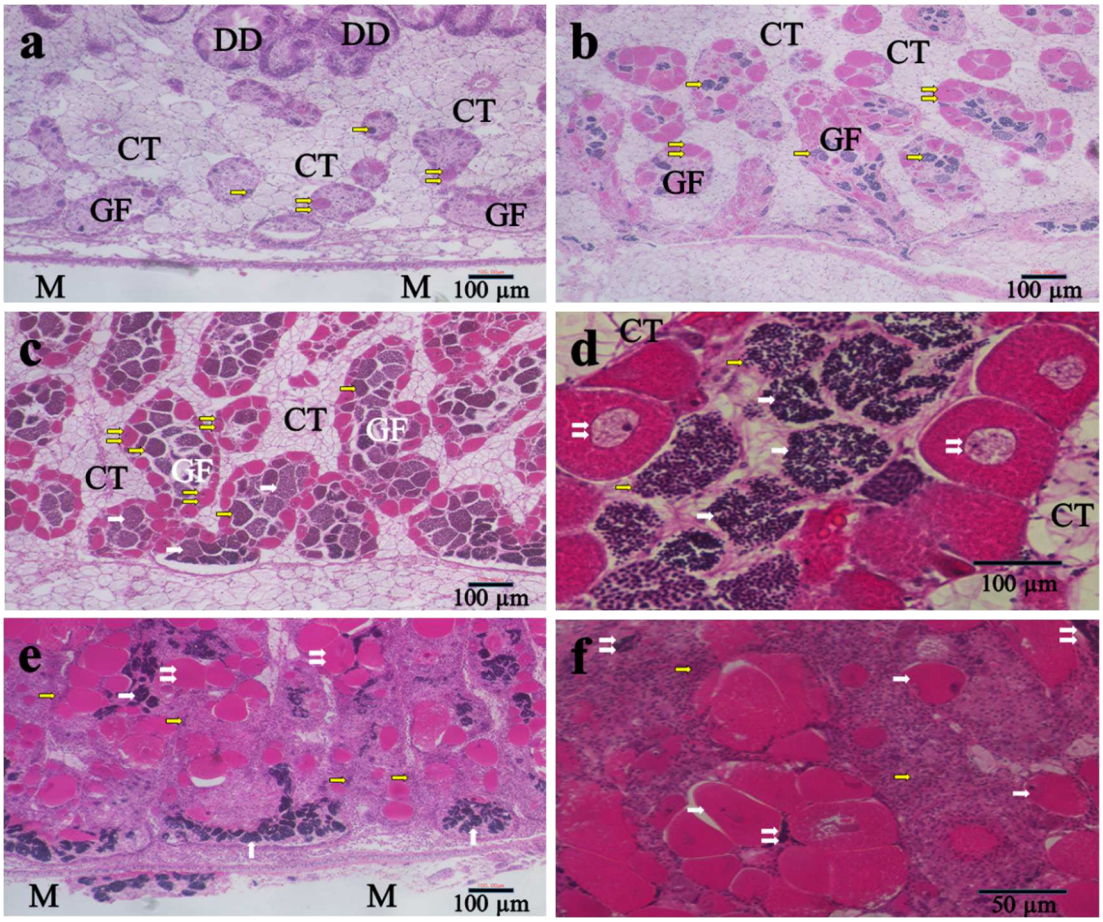

Fig. 2: Histological sections of hermaphroditic *Ostrea angasi* at different stages of gonad development. a) inactive or resting gonad, single and double arrows indicate rudimentary spermatogonia and oocytes, respectively. b) early gametogenesis, single and double arrows indicate developing spermatogonia and oocytes, respectively. c) advanced gametogenesis. Presence of spermatocyte (white arrow), spermatid (single yellow arrow) and medium sized oocytes (double yellow arrow) in the gonad follicles. d) mature gonad. Presence of spermatocyte (yellow arrow), spermatid (single white arrow) and mature eggs (double white arrow) in the gonad follicles. e) Partially spawned gonad. Presence of residual spermatids (single white arrow), eggs (double white arrow) and phagocytes (yellow arrow). f) Partially spawned gonad. Presence of residual spermatids (double white arrow), eggs (single white arrow) and phagocytes

(yellow arrow). CT: connective tissue; DD: digestive diverticula; GF: gonad follicle; M: mantle.

3.3.4 Larvae occurrence and sex ratio

A total of 60 oysters were opened each month to count larvae-bearing oysters. This study adopted the classification of oyster larvae as white-sic, grey-sic and black-sic from Carson (2010). Identification of sex category (male, female, hermaphrodite or undifferentiated sex) was based on gonad histology and microscopic observation of 840 oysters from April 2014 to May 2015. Unless the presence of spermatozeugmata, larvae-bearing oyster was considered a female.

3.3.5 Biochemical composition

The gonad-visceral tissues were the material for analyses of glycogen, protein and lipid contents because oyster gonad anatomically merged with the digestive system. Tissues from ten live oysters were collected in a replicate and three replicate per sampling, freeze-dried for 48 hours, and stored at -80 ^{o}C for no more than three months prior to analysis. Glycogen, protein and lipid contents were determined according to the modified Anthrone technique (Roe and Dailey, 1966), Coomassie Bradford assay (Bradford, 1976) and modified Bligh Dyer method (Folch et al., 1957), respectively.

3.3.6 Condition index

Prior to condition index analysis, oysters were cleaned by scrubbing off barnacles and debris, blotted dry, opened shells, freeze-dried shells and soft tissues for 48 hours, and measured weights using an analytical scale (0.01 g). The condition index (CI) was calculated with the formula: $\mathrm{CI} = \frac{\text{dry flesh weight}}{\text{dry shell weight}} \times 100$

3.3.7 Data calculation and statistical analysis

To calculate gonad maturity index, oysters were ranked from 1 to 4 based on the gonad development stage. The rank of inactive, resting and spawned gonads was 1; early gametogenesis was 2; advanced gametogenesis was 3; and mature gonad was 4. The calculation of gonad maturity index (GMI) used the formula of $\mathrm{GMI} = \frac{\Sigma\,(\mathrm{n} \times \mathrm{F})}{\mathrm{N}}$ (Vaschenko et al., 2013), where, n is the number of oysters at certain gonad stage F (1-4 score) and N is the total number of oysters in the sample. Pearson correlation was applied to measure the relationships of (a) gonad maturity index vs biochemical compositions and condition index, and (b) temperature vs chlorophyll *a*. One-way repeated measures ANOVA was the method to determine monthly variations of (a) chlorophyll *a*, (b) biochemical compositions and (c) condition index. Duncan multiple range test was used to compare the differences between treatment levels when the effect of main treatment factor was statistically significant at $P < 0.05$. All the data were analyzed using the SPSS version 23 (IBM Corporation, Armonk, NY, USA).

3.4 Results

3.4.1 Environmental parameters

Seawater temperature gradually decreased from 19.3 °C in April to 11.2 °C in July 2014 and increased from 12.0 °C in August 2014 to 24.2 °C in February 2015 (Fig. 3a). Salinity was relatively stable but decreased from 41.7 ppt in April to 36.5 ppt in July 2014 and then increased from 36.8 ppt in August 2014 to 41.3 ppt in April 2015. The monthly differences in chlorophyll *a* contents were significant ($P < 0.0001$). Chlorophyll *a* decreased from 1.27 mg L^{-1} in April to a minimum of 0.92 mg L^{-1} in July 2014 and then increased to a maximum of 1.95 mg L^{-1} in January 2015 (Fig. 3b).

Seawater temperature and chlorophyll *a* were positively correlated (r = 0.74, $P < 0.0001$).

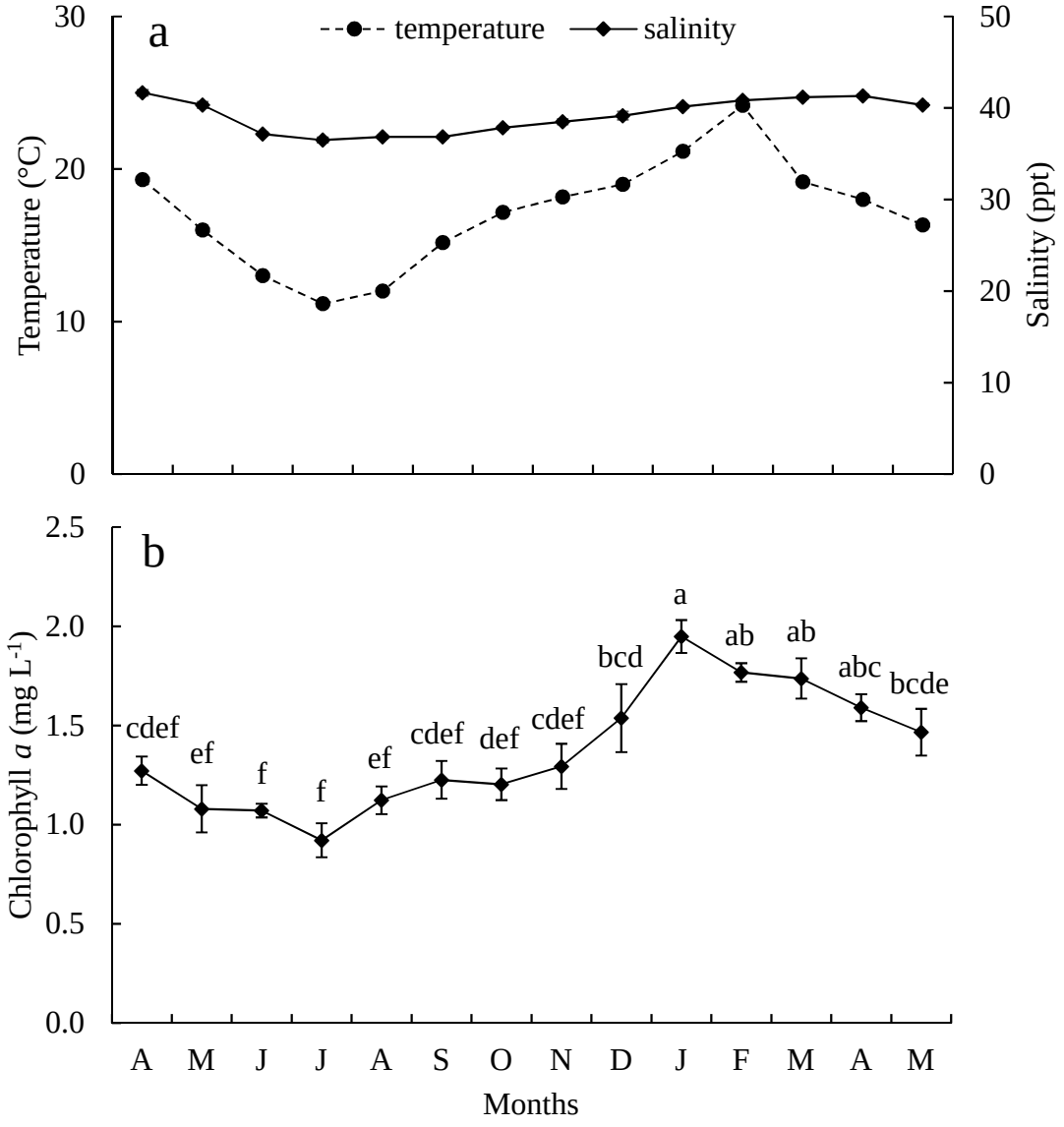

Fig. 3: The monthly variations of environmental parameters (a) seawater temperature and salinity, and (b) chlorophyll *a* in Coffin Bay, South Australia from April 2014 to May 2015. Different letters indicate significant differences ($P < 0.0001$) among the monthly values. Each bar represents mean ± SE of three replicates.

3.4.2 Gametogenesis

Spermatogenesis involved the formation of encapsulated spermatozeugmata. The same oysters carried different development stages of spermatozeugmata, i.e., presence of spermatogonia, spermatocytes and spermatids at the advanced spermatogenesis stage (Fig. 1e). At the start of oogenesis, gonad follicle consisted of only a few oogonia (Fig. 1b) but the oogonial cells gradually aggregated and became larger in the next stage (Fig. 1d). The same individual carried similar size oocytes, and the oocytes matured at a similar time (Fig. 1h). Vitellogenesis, yolk deposition in the ooplasm, started at stage 3 and continued until the eggs fully matured. In a hermaphroditic gonad, the gametes of each sex were in a similar development stage but the gonad follicles of the partially spawned individuals indicated that spermatozeugmata were released before egg ovulation (Fig. 2e and 2f). The inflated empty gonad follicles indicated complete absorption of residual gametes after spawning.

3.4.3 Larvae occurrence

This species carried larvae in the mantle cavity from May to December. The percentage of larvae-bearing oysters gradually increased from 2.5% in May to a maximum of 10% in October and decreased to 3.3% in December (Fig. 4b).

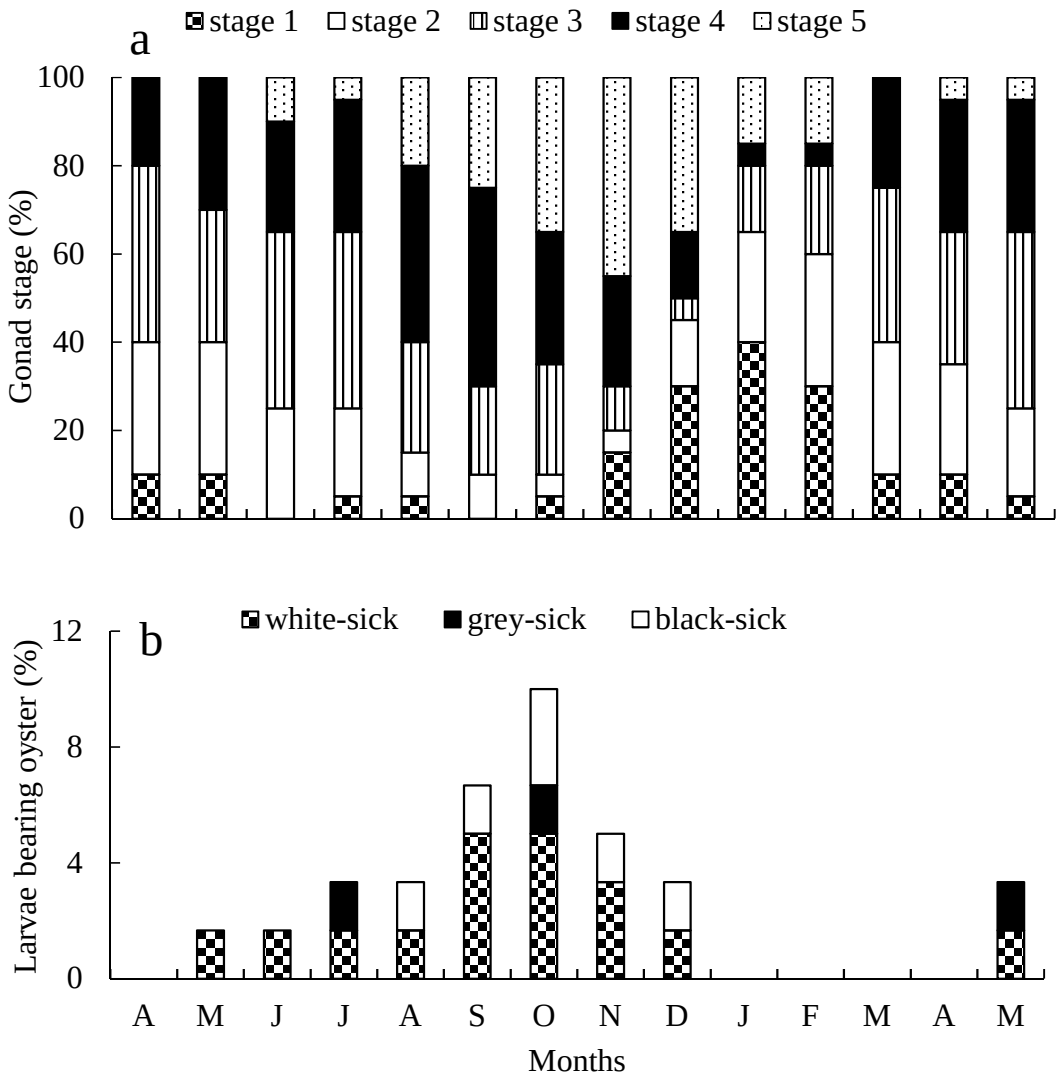

Fig. 4: The monthly variations of (a) gonad development stages, and (b) frequency of larvae-bearing *Ostrea angasi* collected from Coffin Bay, South Australia from April 2014 to May 2015.

3.4.4 Spawning periodicity

Gonad histology revealed an active gametogenesis throughout the year in this species (Fig 4a). However, both gonad histology and larvae-bearing oysters indicated a spawning period from May to December with a relatively higher spawning intensity from September to December (Fig. 4a and 4b).

3.4.5 Sex ratio

The percentages of males, females, hermaphrodites and undifferentiated sexes were 41.3%, 5.8%, 46.7% and 6.2%, respectively (Fig. 5). The male to female ratio was 7:1. In hermaphroditic gonads, male and female gamete proportions ranged from highly skewed to one sex to similar proportions in both sexes.

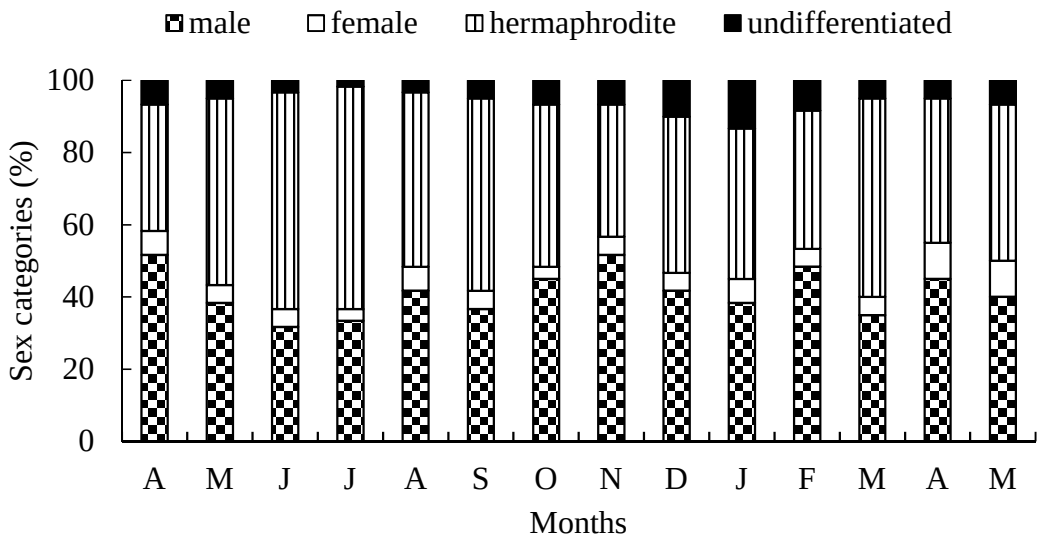

Fig. 5: The percentage of different sex categories of *Ostrea angasi* collected from Coffin Bay, South Australia from April 2014 to May 2015. Sixty oysters were observed each month to determine sex categories.

3.4.6 Dynamics in biochemical composition

The monthly differences in glycogen ($P < 0.0001$), protein ($P = 0.0011$) and lipid ($P < 0.0001$) contents were significant. The glycogen content increased from 171.7 mg g^{-1} in April to 217.5 mg g^{-1} in August and subsequently decreased to a minimum of 106.9 mg g^{-1} in December. The glycogen content again increased from 140.9 mg g^{-1} in January to a maximum of 252.7 mg g^{-1} in May. The protein content increased from 320.7 mg g^{-1} in April to a maximum of 338.8 mg g^{-1} in August and decreased to a minimum of 258.5 mg g^{-1} in February. In the following months, the protein content further increased up to

324.3 mg g^{-1} in May. The lipid content was relatively stable but increased from 136.5 mg g^{-1} in April to a maximum of 146.2 mg g^{-1} in August, and subsequently decreased to a minimum of 113.5 mg g^{-1} in December (Fig. 6a). The gonad maturity index was positively correlated with glycogen (r = 0.70; *P* = 0.0052), protein (r = 0.63; *P* = 0.016) and lipid (r = 0.91; *P* < 0.0001). Chlorophyll *a* was negatively correlated with protein (r = -0.51; *P* = 0.00055) and lipid (r = -0.55; *P* = 0.00016) but the correlation between chlorophyll *a* and glycogen was not significant (r = 0.063; *P* = 0.69).

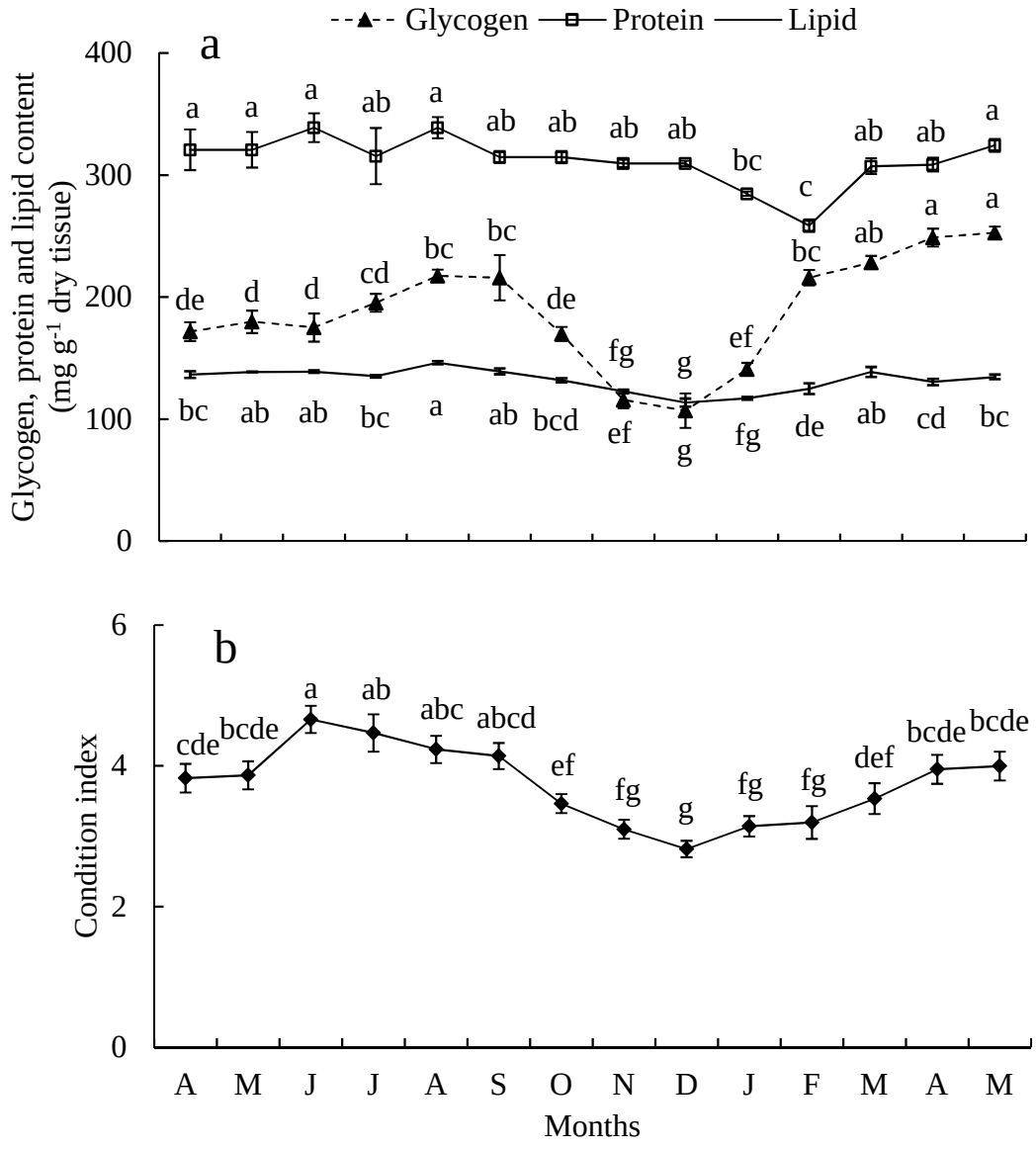

Fig. 6: The monthly variations of (a) glycogen, protein and lipid content (mg g^{-1} dry tissue) in the gonad-visceral tissues, and (b) condition index of *Ostrea angasi* from April 2014 to May 2015. (a) Tissues from ten individuals were pooled in a replicate and each bar represents mean ± SE of three replicates. Different letters within each row indicate significant differences among the monthly values of glycogen (middle row; $P < 0.0001$) protein (top row, $P = 0.0011$) and lipid (bottom row; $P < 0.0001$) contents. (b) Each bar represents mean ± SE of twenty individuals. Different letters indicate significant monthly differences in condition index ($P < 0.0001$).

3.4.7 Dynamics in condition index

The monthly variations in oyster condition index were significant ($P < 0.0001$). The condition index increased from 3.8 in April to a maximum of 4.7 in July and decreased to a minimum of 2.8 in December (Fig. 6b). In the following months, the condition index gradually increased up to 4.0 in May. The condition index was positively correlated with gonad maturity index (r = 0.87; $P < 0.0001$), glycogen (r = 0.54; $P = 0.045$), protein (r = 0.65; $P = 0.011$) and lipid (r = 0.82; $P = 0.00036$).

3.5 Discussion

During spermatogenesis, the existence of spermatozeugmata at different developmental stages indicates that the same individual can repeatedly produce mature spermatozeugmata. In contrary, the similarity of egg developmental stage within the same individual indicates synchronous ovulation of eggs. Although the development stages of both male and female gametes were similar in hermaphrodites, the partially spawned individuals reveal that spermatozeugmata are released before eggs are ovulated.

In spermcasting species, the differences in time window between egg ovulation and spermatozeugmata release determine fertilization success. Egg fertilization requires acquisition of functional spermatozeugmata inside the female/hermaphrodite mantle cavity and earlier spawning of spermatozeugmata ensures a time window within which spermatozeugmata can be acquired. In flat oysters, spermatozeugmata maintain functionality up to 24 hours after release in seawater (Hassan et al., 2016), and successful fertilization would be possible if a female/hermaphrodite acquires them within this period. However, if eggs are ovulated before spermatozeugmata acquisition, the egg fertilization or embryonic development may fail. In a spermcasting ascidian, when insemination was delayed experimentally the majority of oocytes was fertilized but the resultant embryo failed to complete the development (Stewart-Savage et al., 2001). Spermatozeugmata may also bring a cue for females or hermaphrodites to initiate ovulation process as early spawning of sperm could trigger egg ovulation in ascidian *Diplosoma listerianum* and bryozoan *Celleporella hyaline* (Bishop, 2000). However further studies are required to understand if this process also exists in the spermcasting oysters.

As spermatozeugmata could only survive within a certain period, the density of brooders in the population is critical to ensure effective acquisition of spermatozeugmata. The increased distance between male and female brooders may reduce chance for female to acquire spermatozeugmata as they could sink to the bottom, drift away from the female or lose motility due to energy depletion. A low density among brooders has been showed to reduce fertilization success in the scallop *Pecten fumatus* (Mendo et al., 2014) and the distance >50 cm between spawning scallop *Chlamys Bifrons* could result in unsuccessful fertilization (Styan, 1999). The potential brooder distance impact on egg fertilization was minimised in some spermcasting

oysters. For example, the females in *O. puelchana* deploy a strategy by attracting dwarf males on the shell (Pascual, 1997).

The temporal pattern of gametogenesis and spawning might be species-specific among spermcasting oysters. In addition, the same species originated from different geographic location may also differ in gametogenesis pattern. For example, *O. edulis* of Irish and Greek origin showed significant differences in the temporal pattern of gametogenesis (da Silva et al., 2009). In *O. angasi* collected from South Australia, gametogenesis took place throughout the year but spawning occurred for a period of 8-months. Year-round gametogenesis was also observed in *O. nomades* but the spawning time was remarkably irregular among different years (Siddiqui and Ahmed, 2002). In other species, spawning occurred for 8-months period in *O. chilensis* (Brown et al., 2010) and 4-months period in *O. puelchana* (Castaños et al., 2005).

In this study, the sex ratio of *O. angasi* was highly skewed to males and hermaphrodites. The high percentage of males (41.3%) can be explained by the fact that the average size of oysters used in this study was 77 mm (2-3 years of age), as it is common for oysters to firstly mature as the male (Mazón Suástegui et al., 2011). The high percentage of hermaphroditic individuals (46.7%) in *O. angasi* might be an adaptive strategy to use either the male or female function to increase the mating opportunity in a female liming population. In the European flat oyster *O. edulis*, 43% males and 37% hermaphrodites at the size of >5 cm (Acarli et al., 2015) suggest a generality of high percentage of hermaphrodites in an early spawning population of spermcasting oyster species. In contrast, very low percentage of hermaphrodites (2%) and high percentage of males (80%) in a population of *O. edulis* limited the opportunity for maternal role, and rendered recruitment failure in the Solent, UK (Kamphausen et al., 2011).

A brief review on the sex ratio of two genera (*Crassostrea* for broadcasting oysters and *Ostrea* for spermcasting oysters) in Ostreidae reveals that the sex ratio of the broadcast spawners and spermcast spawners are different (Table 1). The percentage of hermaphroditic individuals in broadcast spawners is about 1% (Steele and Mulcahy, 1999; Enríquez-Díaz et al., 2009; Castilho-Westphal et al., 2015) whereas in spermcast spawners is over 35% (Acarli et al., 2015; this study) at the similar size range between these two genera. The contrasting sex ratios between oysters in different taxonomic groups would provide a hint to understand the regulation of sex ratio in relation to spawning strategy of broadcasting and spermcasting oysters.

Table 1: A comparison of sex ratio between two oyster genera (*Crassostrea* and *Ostrea*) that represents broadcast and spermcast spawners.

Reproductive strategy	Scientific name	Geographic location	Oyster age and size	Male to female ratio	Hermaphrodite occurrence	References
Broadcast spawning	*Crassostrea gigas*	Dungarvan and Cork Harbour, Ireland	2 years; 9.2 cm	1:1	< 1%	Steele and Mulcahy, 1999
		Northern Patagonia, Argentina	> 4 cm	1:1	None	Castaños et al., 2009
		English Channel and bay of Biscaye, French	1-2 years	1.69:1 and 1.22:1	< 1%	Enríquez-Díaz et al., 2009
		Gulf of Tunis and Bizert lagoon, Tunisia	8-10 cm	1.4:1	< 1%	Dridi et al., 2014
	C. angulata	Western coast of Taiwan island	10-15 cm	1:0.9	4.2%	Vaschenko et al., 2013
	C. corteziensis	Coastal lagoon in Northwest Mexico	8-10.3 cm	1:3	None	Rodríguez-Jaramillo et al., 2008

	C. brasiliana	Paraná, Brazil	1.1-9.4 cm	1:2.65	1%	Castilho-Westphal et al., 2015
Spermcast spawning	*Ostrea edulis*	Solent, United Kingdom	4-6 years; 5-7 cm	6:1	2%	Kamphausen et al., 2011
		Izmir Bay, Turkey	> 5 cm	19.3:1	37%	Acarli et al., 2015
	O. angasi	Coffin Bay, South Australia	2-3 years; 6.4-8.9 cm	7:1	46.7%	This study

Gametogenesis affects energy storage status and body condition index (Vite-García and Saucedo, 2008; Karray et al., 2015), which is also supported by this study as the gonad maturity index was positively correlated to glycogen, protein and lipid contents, and the condition index. However, the negative correlations of protein and lipid with chlorophyll *a* involve complex interactions in this study. The food availability and spawning intensity are positively related to temperature elevation, but the increase of spawning activity decreases energy storage in oysters (Newell and Branch, 1980), resulting negative correlations of protein and lipid with chlorophyll *a* in *O. angasi*.

Marine bivalves allocate energy to gametogenesis by metabolizing biochemical compounds such as glycogen, protein and lipid, but the strategy to metabolize each compound is species-specific. Glycogen serves as the main energy reserve in most bivalves, but protein and lipid are also used as an additional or alternative energy source (Mathieu and Lubet, 1993). The high variation in glycogen content between pre-spawning, spawning and post-spawning oysters suggests that glycogen serves as the main energy source for gametogenesis in *O. angasi*. However, this species could use protein as an additional energy source especially when the level of glycogen reserve become low. Like this species, both glycogen and protein are metabolized to supply energy for gametogenesis in other bivalves such as *C. gigas* (Dridi et al., 2007), *Mactra veneriformis* (Ke and Li, 2013), *Perna picta* (Shafee, 1989) and *Atrina japonica* (Lee et al., 2015). The consistent lipid content throughout the year indicates minimal lipid metabolization for gametogenesis in *O. angasi*, although lipid metabolization is the main energy source in *O. edulis* (Ruiz et al., 1992).

The energy required for gametogenesis in *O. angasi* was derived from both stored energy metabolism and food intake. The periods of energy storage and energy utilization for gametogenesis overlapped from March to September, thereby this species

would use an intermediate energy metabolism strategy of conservative and opportunistic species. The intermediate energy metabolism strategy is also adopted by other bivalves such as *P. perna* (Benomar et al., 2010), *M. veneriformis* (Ke and Li, 2013) and *Pteria sterna* (Vite-García and Saucedo, 2008) although none of the species has a protracted spawning period like *O. angasi*. In contrast, spermcasting *O. edulis* use opportunistic energy metabolism strategy in which energy supply for gametogenesis is predominantly sourced from food intake (Ruiz et al., 1992). The variation of energy metabolism between congener *O. edulis* and *O. angasi* in different geographical locations suggests that the strategy for energy metabolism depends on species and environment conditions. Interestingly, energy provision from degenerated eggs is another energy retrieval strategy for a protracted spawner *Pecten fumatus* during a period of low food availability (Mendo et al., 2016). The inflated empty gonad follicles suggest that *O. angasi* could resorb the unspawned gametes but future study is needed to understand the pathways of energy allocation from degenerated gametes towards the next round of gametogenesis.

In conclusion, the asynchronous development of spermatozeugmata and synchronous development of eggs favours fertilization success in spermcasting species. The high percentage of hermaphrodites is an adaptive strategy to increase mating opportunity by taking the role of either male or female. The intermediate energy storage strategy between conservative and opportunistic species is a further adaptation to use both conserved energy and consumed food to ensure energy supply for gametogenesis. The asynchronous sperm development pattern, skewed sex ratio and intermediate energy metabolism are adaptive strategies to increase reproductive success of spermcasting molluscan species.

3.6 Acknowledgements

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Chapter 4: Sperm cryopreservation in oysters: a review of its current status and potentials for future application in aquaculture

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4.1 Abstract

Cryopreservation has been expected to improve the efficiency of hatchery operation by supplying gametes on demand without live broodstocks and genetic improvement programs by achieving desired mating and establishing across-generation controls. The preservation of genetic materials of improved stocks and the original population is immensely important for oyster aquaculture industry to prepare the potential impacts from epidemic diseases and natural disasters. This review summarizes the research progress of sperm cryopreservation in oysters and discusses the scope of application of preserved sperm in aquaculture. A bulk of about 50 reports have been published on oyster sperm cryopreservation since 1971, nevertheless the application of this technique in aquaculture is limited. These studies primarily focused on the development of protocol for individual species by optimizing a set of interacting variables at different steps from sperm collection to post-thawing and fertilization. A number of approaches such as sperm motility, morphological integrity and fertility are used to evaluate the cryopreserved sperm quality but there are considerable variations in practice. We synthesized the outcomes in the existing literature in an attempt to suggest the standardization of sperm cryopreservation technique and provide directions for future research.

Keywords: Sperm, Cryopreservation, Oyster, Aquaculture

4.2 Introduction

Cryopreservation is the preservation of living cells and tissues in liquid nitrogen (LN) at –196 ^{o}C for a longer period without compromising biological functionality. This technique offers an opportunity to transport living cells to multiple locations with less disease or biosecurity concerns because frozen cell is less likely to carry diseases than a whole organism. As cryopreservation has wider applications in aquaculture, biotechnology, ecotoxicology and basic research (Chen et al., 1998; Paredes and Bellas, 2009), there is a need to develop a reliable method for sperm cryobanking to meet the demand of different applications (Lubzens et al., 1997). The earliest successful cryopreservation is reported by Luyet and Hodapp (1938) in frog sperm and Jahnel (1938) in human sperm. A major breakthrough came after the use of glycerol as a cryoprotective agent in human sperm cryopreservation (Polge et al., 1949). In livestock, sperm cryopreservation is a proven technique for developing, maintaining and distributing genetic materials. Since the first report of Pacific herring sperm cryopreservation in 1953 (Blaxter, 1953), the protocols have been developed for more than 200 aquatic species around the world (Gwo, 2000; Tiersch, 2000), and many more in the last decade.

Along with terrestrial vertebrates and fishes, molluscs have received attention in cryopreservation studies including oysters (Dong et al., 2005b; Adams et al., 2008; Yang et al., 2012), mussels (Di Matteo et al., 2009; Smith et al., 2012), scallops (Yang et al., 2007; Espinoza et al., 2010), pearl oysters (Acosta-Salmón et al., 2007;

Kawamoto et al., 2007), clams (Dupré and Guerrero, 2011) and abalones (Zhu et al., 2014). While sperm is noted for a wider range of studies, the eggs, embryos and larvae are also considered for cryopreservation in these species (Chao et al., 1997; Paniagua-chavez et al., 2000; Smith et al., 2001). Among the molluscan species in sperm cryopreservation, oysters are most widely studied with more than 50 published papers (including research articles, thesis, book chapters, short papers, technical reports and abstracts) since the first report on Pacific oyster sperm cryopreservation (Lannan, 1971). The Pacific oyster is most extensively investigated with 70% of the reports having been published on its sperm cryopreservation.

Sperm cryopreservation has become a billion dollar global business for artificial insemination in dairy cattle whereas the technology has been limited to research exploration in oysters (Tiersch et al., 2007). The dairy cattle industry almost entirely depends on cryopreserved sperm for breeding programs thus offering a model for commercial application (Caffey and Tiersch, 2000). The strategies for application of cryopreserved fish sperm for aquaculture are already in place (Tiersch, 2008). Gene bank of cryopreserved sperm for Atlantic salmon, *Salmo salar* is established in Norway to preserve the genetic diversity of the natural stocks (Walso, 1998). Moreover, high throughput application of cryopreserved sperm for processing at commercial scale has already established in fish and oyster (Hu et al., 2011; Yang et al., 2012).

Sperm cryopreservation in oysters has progressed over the last a couple of decades by developing protocols for a number of species, but many issues are still unresolved

for the application of research results. Application of cryopreserved sperm for aquaculture is constrained by technical requirements from research findings to commercial operations. The commercial scale sperm cryopreservation has been evaluated (Dong et al., 2005a; Adams et al., 2009), and the use of existing livestock cryopreservation facility has proven practical for oyster sperm (Dong et al., 2007b). In addition, the application of cryopreserved sperm for selective breeding has been assessed in Pacific oysters (Adams et al., 2008). Considering the biological potentials, we would like to draw attention towards the feasibility of application of cryopreserved oyster sperm in aquaculture.

There are a number of reviews that focused on the sperm cryopreservation in aquatic shellfishes and invertebrates (Gwo, 2000; Chao and Liao, 2001; Tiersch et al., 2007). However, the information on oyster sperm cryopreservation remains scattered in literature. There has been a general lack of standardization among studies, rendering it difficult for the future researcher to reproduce the result (Dong et al., 2011), therefore, standardization of cryopreservation procedure is required to ensure repeatability. Moreover, the feasibility of application of cryopreserved oyster sperm should be discussed to represent the potential of cryopreserved sperm in breeding programs, such as selective breeding, hybridizations through the cross of inbred lines established by self-fertilization and production of triploids. There has been substantial progress in Pacific oyster and eastern oyster sperm cryopreservation and can be used as a model for

other oysters. Furthermore, inclusion of new approaches and improvement of the existing procedure is also desired in future research.

In this review, we attempt to (1) summarize the procedures and the factors affecting sperm quality in cryopreservation (2) discuss the standardization of cryopreservation procedures, (3) explore the feasibility of applications of cryopreserved oyster sperm to selective breeding program in aquaculture, and (4) discuss the directions for future research.

4.3 Procedures in cryopreservation

Cryopreservation procedures involve a series of steps which are optimized by experimental trials. The steps include: 1) sperm collection, 2) selection of extenders for sperm dilution, 3) Choice of cryoprotectants for sperm equilibration and evaluation of toxicity, 4) packaging of sperm, 5) cooling and freezing and 6) thawing. Optimization of a set of interacting variables at each of the steps is crucial in any cryopreservation development. However, all the mentioned steps vary considerably within and among species (Tiersch, 2000). Cryopreservation includes the entire cycle from sperm collection to thawing without major change in functional capability of sperm. Some of the steps (e.g., sperm collection and dilution) in cryopreservation are relatively mild, while the other steps (e.g., cooling, freezing and thawing) are extremely stressful for sperm. The steps in sperm cryopreservation of oysters are discussed as follows.

4.3.1 Sperm collection

The suitability of sperm collection methods for cryopreservation needs critical appraisal based on the merits and purposes of the cryopreservation. Sperm is collected from individual males or pooled from several males during cryopreservation. The pooled sperm would minimize individual variations in sperm quality but it cannot be used in selective breeding programs for a single pair mating. Oyster sperm can be collected by natural spawning (Hughes, 1973; Yang et al., 2013), biopsy with notching or anaesthesia to access gonad (Li, 2009; Yang et al., 2013) and stripping (Dong et al., 2007b; Adams et al., 2008; Yang et al., 2012). The male oysters can be induced to spawn without sacrificing the animal by keeping at 4 °C overnight in air and subsequently increasing water temperature and adding algae after immersion (Wang et al., 2008). Injection of serotonin in the gonadal tissue also induces a mature oyster to release sperm within 30 min (Gibbons and Castagna, 1984). Cryopreservation involves maintenance of sperm at a suitable concentration for sample handling at different stages, but natural spawning fails to yield desired sperm concentration. The method for obtaining sperm should be determined by the purpose of cryopreservation and the case of study.

Notching or applying anaesthesia is another non-lethal sperm collection technique for cryopreservation. Notching is the process of making hole on the shell by a grinding wheel that ensures collection of a desired volume of sperm for cryopreservation but this technique damages mantle tissue and reduce the chances of animal survival. Anaesthetic

agents such as dead sea salt (33.3% $MgCl_2$, 24.3% KCl, 5.5% NaCl, 0.2% $CaCl_2$, 0.5% Br^-, 0.15% sulfates and 36.4% crystallization water) and Epson salt ($MgSO_4 \cdot 7H_2O$) have been used to collect sperm. These salts do not induce spawning activity but help opening of shells which are otherwise tightly closed by the adductor muscles. Dead sea salt is used as an anaesthetic in Pacific oysters (Namba et al., 1995; Suquet et al., 2009), American oysters (Yang et al., 2013), Sydney rock oysters (Butt et al., 2008) and flat oyster (Suquet et al., 2010) with varied effectiveness among species. The Epson salt that is traditionally used in biomedical anaesthesiology has also been effective in opening of oyster shells. Epson salt is found more effective between the two anaesthetic agents during sperm collection in American oysters (Yang et al., 2013). Oysters response asynchronously after applying anaesthesia during sperm collection, which may be an advantage for the application requiring sperm from individual males in selective breeding. The asynchronous response is disadvantageous if the required number of individuals does not response on time. Cryopreservation normally involves sperm from multiple males thus the effect of asynchronous response can be minimized by anesthetizing more broods during sperm collection.

Stripping of gonad is the most widely used method in cryopreservation because of suitability of a required volume of sperm collection, easy to practice and less time consuming. In general, stripping involves inserting of a Pasteur pipette beneath the gonad epithelium to suck sperm from a ripe gonad. The collection of oyster sperm by dissecting gonad is also termed as stripping. The dissected gonad is placed in seawater

and the suspension is filtered through a 15-40 μm screen to separate the chunks of gonad tissues from sperm. Large volume of sperm can be collected by gonad dissection and the method is suitable for cryopreservation. Although stripping is a fast and efficient method for sperm collection, the animals have to sacrifice. Variation in initial sperm quality due to gonad development stages is another key weakness of this technique.

4.3.2 Selection of extenders for sperm dilution

Extender is a balanced salt buffer solution used to suspend sperm during the preparation of sperm cocktail for cryopreservation. Extenders can be sterilized with or without the addition of an antibiotic. The bacterial growth can affect sperm survival during short-term cold storage in a variety of taxa. Antibiotic reduces bacterial growth and improves sperm viability, but it can be toxic to sperm, and dosage must be optimized prior to use. Antibiotic supplementation would be important if the collected sperm need to be delivered to another place for cryopreservation. Otherwise, the use of antibiotics in cryopreserved sperm is not desirable as bacteria cannot multiply in liquid nitrogen. The most commonly used extenders are seawater (natural, sterilized or filtered, artificial), Hanks' balanced salt solution (HBSS) and calcium-free HBSS. Calcium induces acrosome reaction and agglutination of oyster sperm, therefore, sperm can retain high motility in calcium-free HBSS (Paniagua-Chavez et al., 1998). Bougrier and

Rabenomanana (1986) used a sperm diluent, DCSB4 for Pacific oyster sperm without any further details and no other study followed up the extender.

In freshwater teleosts, sperm is diluted in extender to mimic the osmolality of seminal plasma and immobilize sperm, as the duration of sperm movement is short. In contrary, the duration of oyster sperm movement is several hours to days, therefore, extenders are not used to immobilize sperm. In general, hypotonic exposure of sperm to external medium activates motility in freshwater species whereas the opposite happens to the marine species. Although the osmolality of seminal fluid is similar to that of seawater, oyster sperm is activated in seawater suggesting that osmotic pressure is not involved in sperm motility activation which is a contrasting process with teleost sperm (Suquet et al., 2012). However, a non-isotonic fixation leads to deformities such as formation of tail vesicles, detached tails, and membrane rupture (Dong et al., 2006b). Dilution of sperm a non-isotonic solution can produce misleading interpretation regarding a structural defect because the deformity caused by the osmolality of fixation solution might be interpreted as a crypreservation process. Pacific oyster sperm is isotonic at 800 to 1086 mOsmol kg^{-1} (Dong et al., 2006b) whereas eastern oyster sperm is isotonic at an osmolality of 650 mOsmol kg^{-1} (Yang et al., 2013). Consequently, the osmolality of seminal plasma and extender should be known for sperm dilution.

The choice of dilution ratio of sperm to extender needs careful appraisal because sperm density affects cryoprotectant equilibration and cooling. A range of dilution ratios of sperm to extender has been used in cryopreservation starting from 1:1 to 1:100

(Paniagua-Chavez and Tiersch 2001; Dong et al., 2005a; Vitiello et al., 2011). Since low motility of sperm is observed at higher dilution, a lower dilution ratio of sperm and extender up to 1:3 is suggested for eastern oyster sperm (Paniagua-Chavez et al., 1998). There has been a wide variation in concentration of freshly collected sperm, therefore, a final density of sperm in the suspension should be known irrespective of the dilution ratio.

4.3.3 Choice of cryoprotectants for sperm equilibration and evaluation of toxicity

The choice of a cryoprotectant depends on the delicate balance between its toxicity and capacity to protect sperm. A cryoprotectant reduces cell damage by balancing dehydration and ice-crystal formation during cryopreservation (Leung, 1991). The equilibration is the exposure of sperm to a cryoprotectant solution to balance solute influx and water outflow so that the cells can minimize ice formation and/or shrinkage during cryopreservation. There are variations in the equilibration time of oyster sperm from 5 min to 60 min depending on the cryoprotectant type and concentration. The equilibration of sperm with a cryoprotectant is crucial for sperm survival because shorter equilibration might be insufficient to protect sperm from cold shock whereas longer equilibration could exert toxic effect to sperm. The rule of thumb is the higher the cryoprotectant concentration, the shorter the equilibration duration or vice versa. Identification of the point of equilibration and toxicity tolerance of sperm is important for selection of a cryoprotectant. Cryoprotectants prevent damage during cooling but

exert toxic effect on sperm at higher concentrations, therefore, a cryoprotectant having low toxicity and high permeability is desirable (Tiersch, 2000).

There are functional differences between cryoprotectants depending on their types (e.g., permeating or non-permeating to sperm). During cooling, non-permeating cryoprotectants like sugar or polymer stabilize sperm membrane (Meryman, 1971), but permeating cryoprotectants enter sperm to balance the solute influx or water outflow. Single cryoprotectant as well as a combination of permeating and non-permeating cryoprotectants has been used for oyster sperm cryopreservation (Paniagua-Chavez and Tiersch 2001; Dong et al., 2007b). The non-permeating cryoprotectants such as glycine and trehalose are most commonly used in combination with permeating cryoprotectants.

A combination of permeating and non-permeating cryoprotectants can improve sperm viability by (1) regulating membrane fluidity with more cohesive sperm membrane and (2) increasing the membrane hydrophobicity resulting minimal chance for intracellular ice formation. In oyster, the supplementation of non-permeating cryoprotectant along with permeating cryoprotectant improves sperm fertility. For example, the inclusion of 0.45 M trehalose with dimethyl sulfoxide (DMSO) yields higher fertilization than DMSO alone in Pacific oyster (Adams et al., 2004). The permeating cryoprotectant DMSO is most widely used, and the highest fertilization rate with thawed sperm is achieved at 4 - 20% concentration (Hughes, 1973; Yankson and Moyse, 1991; Yang et al., 2013). DMSO can penetrate cells quickly and gives better protection from freezing and thawing injury. The other permeating cryoprotectants,

glycerol, ethylene glycol, propylene glycol and methanol are also effective at different concentrations (Table 1).

4.3.4 Packaging of sperm

Since the rate of heat transfer during freezing and thawing depends on packaging of sperm samples, selection of straw or container volume is another critical step in cryopreservation. Regardless of the freezing/thawing method, the volume of packaged sperm sample determines the cooling and thawing rates, and this is particularly critical if LN vapour is employed for cryopreservation. Traditionally, 0.25 mL and 0.5 mL French straws offer efficient cooling and thawing (Tiersch et al., 2007), but the use of large volume straw or vial (e.g., 4.5 mL cryovials) has wider application in aquaculture (Adams et al., 2008). The use of large volume straw assumes a trade-off in heterogeneity of temperature inside a straw and the longer freezing point plateau may lead higher percentage of cell damage. Although a 4.5 mL vial has been cooled at 50 °C min^{-1} by a controlled-rate freezer, the sperm inside the vial cools at 9.5 °C min^{-1} (Adams et al., 2004). Sperm is loaded in the straw using a micropipette (pipette tips are cut to fit straws), or drawn by suction. Although sperm loading into straws, sealing the straws, and further loading of straws into a cryobath are done manually in most of studies, the computer controlled system that automatically fill, seal, and label the straws is also used for oyster sperm, allowing greater cryopreservation efficiency in a commercial scale (Yang et al., 2012). The commercial aquaculture involves a large

number of frozen samples that require reliable and quick identification. The suitability of French straws for permanent printing codes assure reliable sample identification allowing the use of cryopreserved sperm in breeding programs at a commercial scale.

4.3.5 Cooling and freezing

Among all the steps in cryopreservation, cooling and freezing are most stressful for sperm because of cold shock and formation of intracellular ice. Identifying suitable cooling and freezing rates is the most critical point in cryopreservation. During lowering temperature from 10 ^{o}C to –16 ^{o}C, cold shock is caused by the increase in membrane tension due to change of membrane lipid from liquid to solid phase. Major injuries occur within 0 ^{o}C to –40 ^{o}C due to formation of intracellular ice-crystal, which is one of the greatest challenges for successful cryopreservation. If ice-crystals are formed outside of the cell, water moves out of cell to balance the salt concentration. To prevent cell damage against the formation of intracellular ice-crystal, a slow freezing rate is ideal, whereas a faster freezing is ideal against the toxic effects of higher solute concentration. As both solute concentration and ice crystal contribute to cell damage, the freezing rate should be fast enough to minimize the exposure time of sperm to extracellular solvents/cryoprotectants and slow enough to minimize the intracellular ice-crystal formation (Tiersch et al., 2007).

Both programmable method such as the controlled-rate freezer and non-programmable method such as LN vapour are used for freezing of oyster sperm.

When the programmable method is used, samples are frozen by a single-step freezing (Dong et al., 2007b; Yang et al., 2012), two-steps freezing (Dong et al., 2006a; Dong et al., 2007a) and multiple-steps freezing (Ieropoli et al. 2004). In the single step freezing, a range of freezing rate is used which is termed as slow freezing rate (<–8 °C min^{-1}), medium freezing rate (–8 °C min^{-1} to –20 °C min^{-1}) and fast freezing rate (>–20 °C min^{-1}). In the two-steps freezing, freezing rate in the first step is slower than the second step. In the multiple-steps freezing employed in one study, the freezing speeds of sperm samples varies at different steps, i.e., (a) –6 °C min^{-1}, (b) –11 °C min^{-1}, (c) –16 °C min^{-1}, and (d) –21 °C min^{-1} (Ieropoli et al. 2004). When the dairy commercial freezing methods are tested for oyster sperm, straws are placed on horizontal racks and frozen at –16 °C min^{-1} to –140 °C in a freezing chamber (Dong et al., 2007b). The viability of oyster sperm in the fore mentioned studies indicates that oyster sperm is able to withstand a wide range of cooling rate during cryopreservation.

During freezing with LN vapour, sperm samples are placed above the surface of LN before being plunged into LN for long-term storage. The freezing rates in LN vapour are affected by the distance between the sperm and the LN surface, exposure time and the volume of a sample. Therefore, a single factor such as height above LN surface cannot be optimized unless the exposure time and sample volume are known. Pacific oyster sperm is frozen by placing straws above 7 cm of LN surface in one study (Kurokura et al., 1990) whereas, 70% sperm survival is achieved by placing straws 17 cm above the LN surface in another study (Yun et al., 2002). In our study with a flat

oyster *Ostrea angasi*, the highest post-thaw motility was achieved when 0.25 ml and 0.5 ml straws were placed at 6 to 14 cm height above the LN surface. Sperm frozen in 0.25 ml straws of the Australian greenlip abalone at 1.3 cm to 5.2 cm above LN surface produced the highest post-thawing motility (Liu et al., 2014). Therefore, there are species specific ranges for optimum rack heights but we assume that oyster sperm can be frozen at a wide range of heights above LN surface irrespective of sperm volume and exposure time.

The controlled-rate freezer is commonly used for sperm cryopreservation research but it involves high initial investment and subsequent maintenance cost. Although the dairy commercial freezing method is not suitable for small scale operation, the system can handle a large number of samples automatically and considered a commercially viable approach. In contrary, LN vapour is easy to handle and offers cost effective application of cryopreservation. This technique is also suggested to establish a sperm cryobank for the aquaculture industry in Australia (Li, 2012).

4.3.6 Thawing

Determining suitable thawing procedure is vital to cryopreservation because sperm can experience stresses due to thawing temperature and recrystallization of water molecule. In general, the thawing rate is faster than the cooling rate (Pegg, 2007) and rapid warming of cryopreserved sperm can prevent recrystallization of internal ice (Mazur, 2004). Thawing is optimized in a range of temperatures from 16 °C to 75 °C

that can be categorized as low (<29 °C), medium (30-49 °C) and high (>50 °C) temperatures. The oyster sperm have been thawed at a range of thawing duration from 2 sec to 2 min, which is also categorized as short (<10 sec), medium (10-30 sec) and long (>30 sec) thawing durations. As the thawing temperature and duration are inversely related, the higher is thawing temperature the shorter is thawing duration or vice-versa. Sperm cell is considered thawed once the ice is melt in the container. In general, sperm samples are placed in a stagnant water bath during thawing. In addition, frozen samples have been thawed with flowing seawater (Yun et al., 2002) or in a stirred water bath (Gwo et al., 2003). A 37-40 °C temperature for 6-8 sec thawing in 0.25-0.5 ml straws can be regarded as a preliminary standard because most of the sperm cryopreservation studies in oysters and fish have optimized thawing protocol at this temperature. The thawing temperature and duration cannot be predetermined unless the sample volume, sperm concentration, container type are known because these factors influence the rate of heat transfer to the cell (Tiersch, 2011).

Table 1: A brief summary of sperm cryopreservation protocols in oysters including key references. CPA, cryoprotective agent; DMSO, dimethyl sulfoxide; EG, ethylene glycol; HBSS, Hanks' balanced salt solution; LN, liquid nitrogen; NR, not reported; PEG, polyethylene glycol; PG, propylene glycol; SW, seawater.

Species	Sperm acquisition and concentration	Extender and CPA	Equilibration (min)	Packaging	Freezing and thawing	Outcomes	References
Crassostrea gigas	notching and biopsy	UV-irradiated SW; 20% DMSO	NR	1 mL ampules	2 min in LN vapour & plunged into LN; NR	up to 10% fertility	Lannan, 1971
	stripping; motility score 4 or 5 (0-5 scale)	UV-irradiated SW; 10-20% DMSO	30	1.8 mL cryotubes	at –4.7° C min^{-1} up to –70 °C and plunge into LN, thawed into water bath at 55 °C for 20 sec	48-93% fertility	Yankson and Moyse, 1991
	stripping;	concentrated polysaccharide	10-30	0.25 mL, 0.5	at –50 °C min^{-1} in LN vapour;	0-100% fertility	Smith et al., 2001

	2×10^{10} mL^{-1}	in distilled water; 5% DMSO		mL and 2.5 mL straws	thawed at 20 °C for 15 sec or 75 °C for 2 sec		
	stripping; >75% motility; 3.7×10^{8} mL^{-1}	artificial SW; 10% DMSO	5	1.5 mL tubes	from room temperature to –30 ℃ at 15 ℃ min^{-1} and plunged into LN, thawed into stirred water bath at 70 ℃ for 1 min	0-40% fertility	Gwo et al., 2003
	stripping; vigorous motility; 1.7×10^{9} mL^{-1}	NR; 5% DMSO + 0.45M trehalose	45	4.5 mL vials	placed in dry ice pellets with methanol at –75 °C for 10 min or 3 cm above LN for 10 min and plunged into LN; thawed at 20 ℃ for 5-8 min	81% fertilization	Adams et al., 2004
	stripping; >80% motility	sterilised SW 10% EG	30	2 mL containers	at –6 °C min^{-1} from 26 °C to –70 °C and plunged into LN;	58.9% D-larvae	Ieropoli et al., 2004

					thawed at 74 ℃ min^{-1} up to 26 °C		
	stripping; motility 57%; 2×10^9 mL^{-1}	calcium-free HBSS; 8% DMSO	25-30	0.5 mL straws	at –16 ℃ min^{-1} up to –140 °C and plunged into LN, thawed at 40 ℃ into water bath for 7 sec	0-96% fertility	Dong et al., 2005a
	stripping; motility 82%; 2×10^9 mL^{-1}	calcium-free HBSS; 2% PG + 6% methanol	10-15	0.5 or 0.25 mL straws	at –5 °C min^{-1} up to –30 °C and at –45 °C min^{-1} up to –80 °C held for 5 min then plunged into LN, thawed at 40 °C into water bath for 6-7 sec	98% fertilization	Dong et al., 2005b
	stripping; motility 64%, 2×10^9 cells mL^{-1}	calcium-free HBSS; 6% PEG + 4% PG or 6% PEG + 4% DMSO	30-60	0.5 or 0.25 mL straws	at –5 °C min^{-1} up to –30 °C and at –45 °C min^{-1} up to –80°C then plunged into LN, thawed at 40 °C into water bath for 6-7 sec	21% fertilization	Dong et al., 2006b
	stripping; motility	calcium-free HBSS;	10-15	0.5 mL straws	at –5 °C min^{-1} up to –30 °C and –45 °C min^{-1}	96% fertilization	Dong et al., 2007a

	90-95%; 2×10^9 cells mL^{-1}	10% methanol or 12% DMSO			up to –80 °C then plunged into LN, thawed at 23 to 25 °C by placing straws on paper towels for 4 min or at 40 °C for 7 sec		
	stripping; 1×10^9 cells mL^{-1}	calcium-free HBSS; 5% methanol or EG	45	0.5 mL straws	at –16 °C min^{-1} up to –140°C and plunged into LN, thawed at 40 °C water bath for 7 sec	49% fertilization	Dong et al., 2007b
	stripping	5% DMSO + 0.54M trehalose	NR	4.5 mL vials	frozen with dry ice up to –75 °C and plunged into LN, thawed at 20 °C water bath for 5 to 8 min	90% fertilization	Adams et al. 2008
C. virginica	natural spawning	filtered SW, 5% and 10% DMSO	NR	2 mL ampule	at –1 °C min^{-1} up to –8°C, –5.5 °C min^{-1} from –8 °C to –25 °C and plunged into LN; thawing by exposing ampules at 21 °C	11% fertility	Hughes, 1973

	stripping	2.6×HBSS; 80 mM glycine + 55 mM $NaHCO_3$ + 8% DMSO	5	0.25 mL straws	at –5 to –7.5 °C min^{-1} from 0 °C to –20 °C, at –5 to –13.5 °C min^{-1} from –20 °C to –80 °C and plunged into LN; thawing into water bath at 55 to 60 °C for 10 sec	up to 91% fertility	Zell et al. 1979
	stripping; $1×10^9$ mL^{-1}	calcium-free HBSS; 15% PG	20	5 mL straws	at –2.5 °C min^{-1} from 15 °C to –30 °C then held 5 min and plunged into LN; thawed into water bath at 70 °C for 15 sec	8-131% relative survival	Paniagua-Chavez et al., 2000
	stripping; $4.4×10^8$ mL^{-1}	calcium-free HBSS, 10% PG	20	5 mL straws	at –2.5 °C min^{-1} from 15 °C to –30 °C then held 5 min and plunged into LN; thawed into water bath at 70 °C for 15 sec	57% fertilization	Paniagua-Chavez and Tiersch, 2001
	stripping;	calcium-free HBSS;	20	0.5 mL straws	at –20 °C min^{-1} up to −80 °C and plunged into	77% fertilization	Yang et al., 2012

	2×10^8 mL^{-1}	10% DMSO			LN; thawed at 40 °C for 8 sec		
	nonlethal collection; 2×10^8 mL^{-1}	calcium-free HBSS; 20% DMSO	20	0.5 mL straws	at –20 to –25°C min^{-1} up to –80°C and plunged into LN; thawed at 40 °C in water bath for 7 to 8 sec	20% fertilization	Yang et al., 2013
C. iredalei	stripping; motility score 4 or 5 (0-5 scale)	UV-irradiated SW; 10-20% DMSO	30	1.8 mL cryotubes	at –4.7 °C min^{-1} up to -70 °C and plunged into LN; thawed into water bath at 55 °C for 20 sec	11-35% motility	Yankson and Moyse, 1991
C. tulipa	stripping; motility score 4 or 5 (0-5 scale)	UV-irradiated SW; 10-20% DMSO	30	1.8 mL cryotubes	at –4.7 °C min^{-1} up to -70 °C and plunged into LN; thawed into water bath at 55 °C for 20 sec	0-71% fertility	Yankson and Moyse, 1991
Saccostrea cucullata	stripping; motility score 4-5 (0-5 scale)	UV-irradiated SW; 10-20% DMSO	30	1.8 mL cryotubes	at –4.7 °C min^{-1} up to -70 °C and plunged into LN; thawed into water bath at 55 °C for 20 sec	0-78% fertility	Yankson and Moyse, 1991
Ostrea edulis	stripping, motility score	filtered SW; 15% EG	10-30	NR	at –3 °C min^{-1} up to –70 °C and plunged into	50% motility	Vitiello et al., 2011

	⩾ 3 (0-5 scale)				LN; thawed into water bath at 55 °C up to 18 °C straw temperature		
	stripping, motility 63%	calcium-free HBSS; 10% DMSO	30	0.5 mL straws	LN vapour for 3 min and plunged into LN, thawed into water bath at 40 °C for 13 sec	8% motility	Horvath et al., 2012

4.4 Standardization of cryopreservation procedure

A general lack of standardization is recognized in the overall sperm cryopreservation procedure in oyster (Dong et al., 2011). There are inconsistencies in reporting of different terminologies, for instance, the percentage of swimming larvae is termed as fertilization rate (Gwo et al., 2003), whereas, the same criterion is also used to calculate hatch rate (Yankson and Moyse, 1991; Dong et al., 2005a). Therefore, standardization of the cryopreservation process is necessary to improve the existing methods. Here, the discussion is focused on the standardization of sperm concentration and quality assessment of oyster sperm because these two factors are most inconsistent in the published protocols causing difficulties to compare the results between studies.

4.4.1 Sperm concentration

Standardization of sperm concentration is crucial for cryopreservation since a cryoprotectant concentration suitable for a sperm concentration may be toxic at a lower sperm concentration. Alongside, agglutination of cryopreserved sperm is a relatively common phenomenon which is primarily found due to lack of sufficient cryoprotectant at a certain sperm concentration. For example, sperm from diploid and tetraploid Pacific oyster showed higher levels of agglutination when DMSO concentration was lower than 12% at sperm concentrations of 2.5×10^{7} to 5×10^{9} cells mL^{-1} (Dong et al., 2007a).

However, higher agglutination of post-thaw sperm does not necessarily lead to lower fertilization rates.

The accurate estimation of sperm concentration is required for a number of reasons: 1) to provide optimal dilution of sperm, 2) to facilitate estimation of cryoprotectant toxicity during equilibration, 3) to prevent sperm agglutination due to lack of optimal cryoprotectant at a given sperm concentration, and 4) to maintain optimal sperm to egg ratio for fertilization. Estimation of sperm concentration by a haemocytometer is considered a gold standard but this technique is not suitable for rapid counting. Cryopreservation requires rapid handling of sperm for further processing, therefore, one of the techniques such as photometry, flow cytometry or the computer assisted sperm analysis (CASA) system can be used to rapid and accurate estimation of sperm concentration. In oyster sperm cryopreservation, a few protocols are developed based on sperm dilution ratio but without optimizing sperm concentration. There are considerable differences of sperm density based on individual, seasonal, animal age and gonad stage variations in the collected sperm. Therefore, sperm concentration must be determined during cryopreservation to ensure reproducibility of a protocol.

4.4.2 Quality assessment of sperm

The standardization of the evaluation of cryopreserved sperm quality is necessary to improve the existing approaches. The quality of sperm can be evaluated based on single

or a combination of multiple criteria, such as a) sperm motility, b) morphological and functional integrity of sperm organelles, and c) fertilization and hatching of eggs.

The biological differences of sperm of broadcasting and spermcasting oyster should be taken into account to evaluate sperm quality. The broadcasting oysters release sperm to fertilize eggs outside the female body cavity, whereas spermcasting species release spermatozeugmata that enter the female mantle cavity to fertilize eggs. In spermatozeugmata, the sperm tails are projected outside while the sperm heads are enveloped by a gelatinous mass (Foighil, 1989). The spermatozeugmata retain structural integrity less than 24 hours and eventually dissociate into motile sperm in the water column. In our study, the dissociation of spermatozeugmata is quicker after the addition of cryoprotectants in flat oyster *Ostrea angasi*. After cryopreservation, the sperm is mostly dissociated from spermatozeugmata but those still attached to spermatozeugmata are non-viable after thawing (Horvath et al., 2012). The frozen thawed sperm quality in flat oyster is assessed by evaluating motility and functional integrity. The sperm quality assessment by fertilization trials is not feasible at the moment due to the lack of a reliable fertilization technique in flat oyster.

Sperm motility is assessed by quantifying the proportion of mobile cells in aliquots of suspension. Sperm movement is categorized as 1) active forward movement, 2) vibration movement, and 3) non movement. Generally forward moving sperm is considered as motile. Either absolute or relative percentage of motile sperm, or motility

at different scales (0-4, 1-4 or 0-5) is used in different studies. Any of the motility estimation can produce reliable assessment if the criteria of motile cells are defined and quantified objectively. The estimation of sperm motility percentage under a microscope by visual observation is a quick and easy method but the subjective observation leads to higher variability. Video recording of sperm movement for motility estimation can provide reliable assessment. A more reliable technique, the CASA system enables quantitative estimation of motility describing the percentage of sperm having curvilinear, straight line, angular and vibration motility (Wilson-Leedy and Ingermann, 2007). The CASA system is a rapid and reproducible technique that can be used to quantify sperm motility with definitive criteria.

The morphological and functional defects of frozen sperm are usually found in plasma membrane, acrosome, mitochondria, midpiece and tail (Kurokura et al., 1990). The ultrastructural studies with a scanning electron microscope (SEM) are useful to investigate the reasons causing low motility and fertility in cryopreserved sperm (Yao et al., 2000). For example, sperm tail damage is associated with lower motility, whereas head damage is related to the development of the embryo. As only a few numbers of sperm out of millions are considered during ultrastructrural studies with SEM, there is a chance of sampling bias. The computer assisted morphology analysis is used to detect morphological alterations of fish sperm after cryopreservation (Peñaranda et al., 2008). The system has advantages over SEM by providing faster and automated sperm

morphology analysis and allowing rapid and reliable identification of sperm defect. More advanced cryo-scanning electron microscopy provides morphological information in a frozen state without sample fixation and is also useful to identify sperm defects (Ekwall, 2009).

Assessment of key sperm organelles such as plasma membrane, acrosome and mitochondria can provide reliable information to explain the damage caused by the cryopreservation process. There are readily available molecular probes that can be used to diagnose sperm defects based on differential staining. For example, live/dead sperm viability kit identifies plasma membrane integrity, LysoTrack green kit identifies acrosome integrity and a combination of Rhodamine and Propidium Iodide identify mitochondrial membrane potentials. Lower motility and fertilization of eggs are caused by the damage to plasma membrane, mitochondria and acrosome of sperm (Paniagua-Chávez et al., 2006). Determination of morphological or functional integrity of thawed sperm provides crucial information of sperm quality, and we suggest incorporations of morphological or functional integrity with other quality assessment indicators to specifically identify the sources of sperm defects.

Although motility, structural and functional integrity are important sperm quality assessment indicators, the ultimate criterion is the ability of sperm to fertilize eggs and subsequent larval survival. Along with sperm, the fertilization rate is also affected by eggs so the egg quality should be ensured during the interpretation of fertilization. If the

number of fertilized eggs with thawed sperm is expressed as absolute percentage, the fertility of fresh sperm must be determined to ensure the consistency of egg quality. Otherwise, a relative fertility percentage (fresh sperm vs thawed sperm) of eggs should be explained. In some of the past literature, quality of frozen thawed sperm is compared based on fertility percentage without mentioning the sperm to egg ratio.

In general, thawed sperm is capable of fertilizing eggs, but a 30 to 100-fold more sperm is required to achieve a similar fertilization rate compared to fresh sperm (Adams et al., 2004). The threshold of using the minimum number of sperm to fertilize an egg can be species-specific and confounded by the fertilisation method. The sperm to egg ratio in of fresh and cryopreserved sperm has been explored to validate the cryopreservation protocol, but the minimum number of cryopreserved sperm required to fertilize an egg has not been rigorously defined. As such variable numbers of the sperm to egg ratio for fertilization are employed while assessing thawed sperm quality from 10-15 to 18,000 sperm per egg. In Pacific oyster, up to 96% fertilization rate was achieved with 10,000 thawed sperm per egg. The fertilization method itself can bias the fertilization rate, so further explanation of the fertilization technique is desirable while assessing sperm to egg ratio. We assume that 1000-10,000 sperm per egg should be applicable to other species to compare fertility if the sperm functionality is not compromised exceptionally by cryopreservation. Fertility assessment must be integrated

with the sperm to egg ratio to provide an indication on the number of straws/vials required for a breeding program to produce desirable number of spat.

4.5 The scope of application of cryopreserved sperm in oyster aquaculture

Oysters are classified into two distinct categories of broadcasting and spermcasting species based on the mode of reproduction (Coe, 1943; Buroker, 1985). Application of cryopreserved sperm in aquaculture has different perspectives in broadcasting and spermcasting species. Cryopreserved sperm can be directly applied in breeding programs in broadcasting oysters whereas it can only act as repository of genetic material at the moment in spermcasting oysters as artificial fertilization has not been investigated in the latter.

4.5.1 Implications of cryopreserved sperm in oyster genetic improvement

Oysters are farmed in Japan from the 15th century while in many other countries such as Australia, Canada, France, Korea, and United States the oyster farming has been practiced almost a century. Among the 40 oyster species, the Pacific oyster is the most important contributor of global molluscan production in aquaculture followed by eastern oyster, rock oyster and flat oyster (FAO, 2012). There has been significant improvement in culture operations because of the development of hatcheries and application of genetic improvement techniques. The wild spat collection for farming is

widespread in areas having natural production but the abundance of wild spat is highly variable because of the environmental uncertainty. The gradual depletion of oyster beds, diseases of wild populations and demand for uniform clutchless seeds are causing a shift towards the hatchery spat for stocking (Robert and Gérard, 1999; Wieland and Economics, 2007). In this scenario, there has been a constant increase of oyster hatchery, leading to the advances in genetic improvement programs such as selective breeding and triploidy.

The selective breeding program can substantially meet the commercial needs of oyster culture attaining faster growth rate and disease resistance. The selective breeding has already demonstrated its potential with 13% average increase in growth rates per generation in a number of fish and shellfishes (Gjedrem, 2002). Selective breeding of Sydney rock oyster has led to 4 - 18% live weight gain in two subsequent generations compared to non-selected controls (Nell et al., 1996, 1999). Likewise, selection has resulted in an increase in 10 - 20% growth rates of Pacific oyster (Langdon et al., 2003; Li et al., 2011), 16 - 39% increase in eastern oyster and 21-42% increase in flat oyster (Newkirk and Haley, 1982) in each generation. Along with better growth rates, selective breeding has yielded oyster strains resistant to a number of diseases such as winter mortality, MSX, dermo and QX diseases (Haskins and Ford, 1988; Vrijenhoek et al., 1990; Calvo et al., 2003; Nell and Perkins, 2006).

In other areas of oyster genetics, production of triploids (3n) has widely been practiced in many parts of the world (Stanley et al., 1981). One of the reliable ways of producing triploids is to cross tetraploid with the normal diploids (Guo et al., 1996; Eudeline et al., 2000) which can be achieved with cryopreserved sperm. The sperm from the tetraploid Pacific oyster has been successfully cryopreserved. As the tetraploid males are not readily available in many parts of the world, cryopreserved sperm from tetraploid males can be transported elsewhere for commercialization of triploids. The farming of triploid oyster has a number of advantages compared to farming diploids for a number of reasons. Oyster gonad occupies almost half of the soft body parts in the reproductive season and after spawning the soft parts become emaciated and watery rendering unsuitable for marketing. On the other hand, the triploids are acceptable to consumers all the year round and offer an extended marketing period. Due to the inability of triploids to spawn, the energy is mobilized for growth and more meat can be produced per individual.

The triploids are produced by applying heat shock or chemical treatment but these techniques have never been successful to produce 100% triploids (Quillet and Panelay, 1986; Downing and Allen Jr 1987; Desrosiers et al., 1993). Tetraploids (4n) are crossed with diploid to produce almost 100% triploids, which has also been adopted by the commercial hatcheries (Guo et al., 1996). Fresh sperm from diploid and tetraploid oysters produced a similar fertilization rate indicating the compatibility of tetraploid

oyster sperm to diploids. In the fertilization trials at 1×10^9 sperm/ml, the cryopreserved sperm from tetraploid oysters produced a significantly lower fertilization rate compared to diploids (Dong et al., 2005a; Dong et al., 2006a). Sperm of tetraploids are generally larger than the diploids so the cryopreservation parameters used for tetraploid sperm might need different factor optimization. Sperm of the tetraploid oysters are either more delicate or have different plasma membrane properties compared to diploids, leading to more sperm damage during sperm freezing and thawing (Dong et al. 2006a). However, this higher sensitivity of tetraploid sperm to cryopreservation can be compensated by increasing the sperm to egg ratio at the fertilization to produce triploids for farming.

Acquisition of cryopreserved sperm offers an important reserve of genetic material that can be used in oyster aquaculture in a number of ways. Cryopreserved sperm allow production of self-fertilized inbred lines in Pacific and eastern oysters after sex reversal from male to female (Lannan, 1971; Li, 2009; Yang et al., 2015). The inbred line is an important system providing opportunities for exploitation of hybrid vigour and genetic research. Cryopreservation has the potential to promote the capacity of selective breeding programs by preserving sperm from improved stock (Adams et al., 2008). When desired mating is required, the stored sperm can contribute to improve the efficiency of a breeding program. Furthermore, the cryopreservation of sperm allows backward selection by providing sperm beyond the lifetime of an individual. The

challenging breeding design to compare the genetic gains across generations is otherwise impossible in natural breeding.

Cryopreservation can protect existing breeding programs of oyster by providing preserved sperm from selected stock. The breeding nucleus can be collapsed because of disease outbreak, natural disaster or anthropogenic activities. The summer mortality, parasitic and viral diseases have caused mass mortality of oyster in the recent past. The oysters have poor immune mechanism compared to the vertebrates (Lacoste et al., 2002), consequently, once the stock is infected, it is intrinsically difficult to control diseases. The frequent collapse of the farm production due to the mass mortality episodes has call for attention to preserve oyster sperm for future. However, the breeding nucleus cannot be reconstructed solely based on cryopreserved sperm unless the acquisition of female broodstock or preserved eggs for fertilization.

4.5.2 Logistics in quality insurance of cryopreserved sperm

During the application of cryopreserved sperm, the main challenge would be maintaining the quality of the frozen sperm after processing of a large number of samples. Short term storage at 4 ^{o}C can also be carried out where sperm cannot be cryopreserved immediately after collection (Vanderhorst et al., 1985). Cryopreservation at a commercial scale involves a large volume of sperm collection, short-term storage, and shipping, and all of these steps are critically as important as basic cryobiological

aspects like freezing and thawing (Tiersch, 2011). Theoretically, the quality of cryopreserved sperm should not change over time but a decrease in sperm viability is found in Pacific oyster sperm after 4 years of storage (Usuki, 2002). An integrated framework is required on sperm cryopreservation considering the strength, weakness and opportunities of the oyster aquaculture industries.

A bulk of research on protocol optimization is already in place, and the future research should include the improvement of integration and efficiency of the cryopreserved sperm for aquaculture. In conjunction with research, private sector involvement to improve current practices would be necessary to protect investment of the entrepreneur by solving disease and biosecurity concerns. Furthermore, cost-benefit analysis, available facilities of the aquaculture industries and the scale of application of the preserved sperm have to be determined prior to application (Caffey and Tiersch, 2000). The cost analysis for selective breeding program using cryopreserved sperm is assessed in Australian aquaculture industry. Using the existing protocol, cryopreserved sperm in a 0.5 ml straw can fertilize 31,250 eggs of Pacific oyster. Theoretically, 84,375,000 fertilized eggs can be produced per 35 L dewar with cryopreserved sperm at $71,416 initial capital investment for oysters (Li, 2012). Along with cost analysis, the marketing of cryopreserved sperm also demands quality control and biosecurity measures.

4.6 Future research

The variation in fresh sperm quality leads to inconsistencies of the outcome of cryopreservation studies so that future research is required to explain the mechanisms controlling sperm quality in oysters. Sperm can be collected by relaxing the adductor muscle but the effect of using anaesthesia on sperm quality is unknown. Generally the sex ratio of oysters is skewed depending on age with more male at younger population or more female at older population. The effect of age or changing sex on sperm quality is not known yet. These factors can bias initial sperm quality, and eventually affect the quality of cryopreserved sperm.

Future research is also desirable to improve the existing cryopreservation procedure. The addition of compounds such as proteins, lipids, vitamins and anti-oxidants have improved post-thawed sperm viability in fish and livestock (Pena et al., 2003; Prathalingam et al., 2006; Martínez-Páramo et al., 2012). We suggest the incorporation of these compounds to test the efficacy of protecting oyster sperm. New approaches such as vitrification should be tested for oyster sperm preservation. As there is no published report, we have limited understanding whether vitrification can be useful to preserve in oyster/aquatic invertebrate sperm. Vitrification is tested with a number of cryoprotectants cocktail in flat oyster *Ostrea angasi* but no post-thaw sperm survival is achieved (unpublished data). Furthermore, the samples plunged in LN after cooling with LN vapour less than 2 cm is also non-viable. There might have species-specific

response to vitrification technique, therefore, further study should be carried out to test whether oyster sperm can withstand ultra-rapid cooling.

4.7 Conclusion

Cryopreservation of oyster sperm has prompted substantial research effort at protocol development and produced some promising results. The integration of past research with a standard approach would lead to greater efficiency of cryopreservation procedure towards expanding application. Cryopreserved sperm has greater applicability in aquaculture through genetic improvement programs of the economically important species. However, there are challenges for aquaculture industries during the transition from research to commercial operation of cryopreserved sperm. Without any doubt, if these challenges are overcome, cryopreserved oyster sperm would become a promising sector in future for the oyster aquaculture development.

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Chapter 5: Development of a spectrophotometric technique for sperm quantification in the spermcasting Australian flat oyster *Ostrea angasi*

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5.1 Abstract

Sperm quantification is vitally important when sperm concentration is required for standardization of different fertilization treatments in a hatchery. Although the haemacytometer method is generally used to determine sperm concentration, the procedure is tedious and the attributes are not suitable for handling a large number of sperm samples within a short period. In this study, the efficiency of sperm concentration determination was improved in the spermcasting oyster *Ostrea angasi* by optimising the regression model and parameters critical to spectrophotometric reading. Although sperm concentration can be estimated in a wide range of wavelengths, the 350 nm wavelength produced the best fit to the regression model ($y = 1\times10^{-8} x + 0.163$; $r^2 = 0.996$). In addition, the sperm counts estimated with this model were similar to the haemacytometer counts. The reading repeatability of this technique was further validated with samples from different individuals. Comparisons with literature suggest that when the spectrophotometric technique is applied to a new species for estimating sperm concentration, the regression relationship between sperm concentration and wavelength reading should be reassessed due to species specific discrepancy.

Keywords: Sperm concentration, spectrophotometry, flat oyster, mollusc

5.2 Introduction

Quantification of sperm concentration is important in aquaculture, especially in breeding programs when standardization of the sperm to egg ratio is required across different fertilization treatments to minimize the sperm density-dependent effect such as abnormal development due to polyspermy (Alliegro and Wright, 1983; Gould and Stephano, 2003) and unsynchronized embryo development due to a lower sperm to egg ratio. In addition, when sperm from multiple males are required for fertilization to

optimize genetic variation, a common practice is to use a similar number of sperm from different males. Standardization of sperm concentration is also one of the important procedures in cryopreservation as sperm concentration is the most uncontrolled variable rendering inconsistent outcome of an established protocol (Tiersch et al., 2006; Dong et al., 2007a; Hassan et al., 2015). The choice of a cryoprotectant concentration is critically linked to sperm concentration. For instance, a cryoprotectant suitable for a certain sperm concentration can be toxic to sperm at a lower concentration (Dong et al., 2007b) or unable to prevent agglutination at a higher sperm concentration (Dong et al., 2007a). Furthermore, the assessment of sperm morphological and functional parameters such as plasma membrane integrity, acrosome integrity and mitochondrial membrane potentials require quantification of cells in the suspension (Gravance et al., 2000). Without sperm concentration, the reagent concentration and incubation time cannot be standardized for fluorescence imaging, and compromise the reliability of the results.

Determination of sperm concentration by a haemacytometer is the basic method and has been considered a gold standard for estimation of accuracy (WHO, 1999). This method is well accepted worldwide with high precision (Bailey et al., 2007). However, the haemacytometer method is tedious and time consuming, requiring 10-15 min to determine the concentration of a sperm sample. Therefore, the method can be impractical for processing a large number of samples, especially in sperm cryopreservation for family based genetic improvement programs. To find a solution to these limitations, a number of sperm counting techniques such as flow cytometry, Coulter counter, Makler counter, and computer-assisted sperm analysis have been developed and applied for sperm quantity and quality analyses in human and economically important animal species (Makler, 1980; Yang et al., 2016). However, these techniques cannot be routinely used in breeding programs of cultured species mainly because of cost and technical limitations. In contrast, estimation of sperm

concentration by spectrophotometry is simple, rapid and reliable, and has been used to facilitate breeding programs and sperm cryopreservation in some bivalve species (Miguel and Beaumont, 2008). The spectrophotometric technique might be species-specific as different absorbance spectra and regression equations have been reported in different bivalves (Del Rio Portilla, 1996), but its suitability to spermcasting species has not been investigated.

Some spermcasting marine species such as the Australian flat oyster *Ostrea angasi* have unique spermatological characteristics (Foighil, 1989; Falese et al., 2011). At spawning, sperm are clustered in spermatozeugmata in which sperm heads are bounded by a gelatinous membrane and tails are extended outside of the membrane (Hassan et al., 2016). The individual sperm then gradually dissociates from spermatozeugmata at various time up to 24 hours. Due to increasing interest in the flat oyster aquaculture in Australia (Heasman et al., 2004; O'connor and Dove, 2009), the establishment of a selective breeding program would be a necessary step for the long-term sustainable development of the industry. To facilitate further development of this core business, this study aimed to develop a spectrophotometric technique for sperm quantification, with the specific objectives (i) to identify the appropriate wavelength in the absorbance spectra and generate a regression line based on sperm concentration and absorbance, (ii) to validate the accuracy of the spectrophotometric technique for estimation of sperm concentrations, and (iii) to test the repeatability of spectrophotometric technique.

5.3 Materials and Methods

5.3.1 The oysters

The Pristine Oyster Farm in Coffin Bay, South Australia provided flat oysters

within the natural spawning season of this species from June to August 2014 by overnight delivery in a chilled Styrofoam box to the South Australian Research and Development Institute (SARDI). The oysters were cleaned off epifauna and placed in an indoor tank in which temperature was maintained at 20 ± 0.5 °C. The tank was supplied with flow-through seawater containing a similar proportion of three mixed microalgae (*Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans*). The oysters used in this study were 2-3 years old, with length and wet weight being 74.9 ± 4.2 mm and 64.3 ± 9.7 g, respectively.

5.3.2 Sperm collection and screening

Sperm were stripped from the gonad using a disposal pipette (3.5 mL, Livingstone International) and placed in 1.5 mL Eppendorf tubes. A 45-μm pore sized mesh was used to separate sperm from gonad tissues and spermatozeugmata. The average diameter of spermatozeugmata is 117 μm (Hassan et al., 2016). The mesh was fitted between two 1-mL pipette tips while the inner tip was cut at the 3 cm from the narrow end. Both pipette tips were fitted to the pipette. The sperm suspension was placed in the inner tip and filtered by a downward push with the pipette. Same volume of sperm pooled from 3-5 males were used in each treatment.

5.3.3 Sperm counting with the haemacytometer method

When this method was applied in this study, the concentration of a sperm sample was determined by an improved Neubauer haemacytometer chamber (Marienfeld, standard depth 0.1 mm). Sperm were diluted 500 times in filtered seawater. An aliquot of 10 μl sperm suspension was loaded, and a coverslip was placed on the chamber. A drop of 1 μl Lugol's iodine was placed on the edge of the coverslip. After 5 min, sperm on the five large squares (area of each square 0.04 mm^2) were

counted diagonally and the sperm concentration was calculated using the following formula: sperm concentration (sperm mL^{-1}) = total number of sperm counted × dilution factor × 4 × 10^6/number of large squares counted. An average of three counts from each sample was used to estimate sperm concentration.

5.3.4 The absorbance spectra and development of regression lines

When original sperm concentrations were determined, the samples were sequentially diluted in filtered seawater to 1.6×10^9, 8×10^8, 4×10^8, 2×10^8, 1×10^8, 5×10^7 and 2.5×10^7 sperm mL^{-1}. The absorbance spectra of known sperm concentrations were generated at the wavelengths of 280-800 nm using a multifunctional microplate reader (CLARIOstar, BMG Labtech, Germany). Filtered seawater was used as a blank during absorbance measurement. Sperm samples were homogenised by tapping the tubes 10 times prior to being pipetted into 96-well flat bottom microplates (Apogent, Denmark). The absorbance was then measured within 10 sec after sperm loading to avoid precipitation. While measuring absorbance, fifty flashes were used per well bi-directionally, from top to bottom and left to right. Four wavelengths 350 nm, 450 nm, 500 nm and 600 nm were chosen to generate regression lines by plotting the haemacytometer sperm counts and the resultant absorbance. The absorbance values between 0.1 to 1.0 were used to generate regression lines.

5.3.5 Validation of the optimised regression line

To validate the accuracy of regression line optimised in the previous experiment, concentration results produced with the haemocytometer method were statistically compared with those generated with the spectrophotometric technique. In this

validation, sperm suspensions were prepared using the same method described in the previous section.

5.3.6 Repeatability of the spectrophotometric technique

To test the repeatability of the technique, the absorbance of sperm suspensions of 5×10^7, 2.5×10^7, 1.25×10^7, 6.25×10^6 and 3.125×10^6 sperm mL^{-1} were measured at the wavelength 350 nm with three sets of samples from three different individual groups. The sperm concentrations calculated by the spectrophotometric technique from different sample sets were compared with the sperm count by the haemocytometer method to test repeatability of the technique.

5.3.7 Statistical analysis

A simple linear regression (SLR) was generated using sperm concentration and resultant absorbance in the equation $y=ax + b$, in which x is sperm concentration and y is absorbance. The coefficient of determination (r^2) was calculated to test the goodness of fit in the regression model. The sperm count by the spectrophotometric technique was compared with the haemacytometer method using independent sample t-tests. The data were analysed with SPSS version 20.0 (IBM Corporation, Armonk, NY, USA).

5.4 Results

5.4.1 The absorbance spectra and regression lines

Figure 1 shows that the absorbance decreased with the increase in wavelengths at all the sperm concentrations assessed but absorbance peaked at different wavelengths. The absorbance peaked at relatively higher wavelengths in less diluted sperm than in more diluted sperm. For instance, the absorbance peaked at 350 nm for the

concentration of 2.5×10^7 sperm mL^{-1} but peaked at 400 nm for the concentration of 2×10^8 sperm mL^{-1} (Fig. 1).

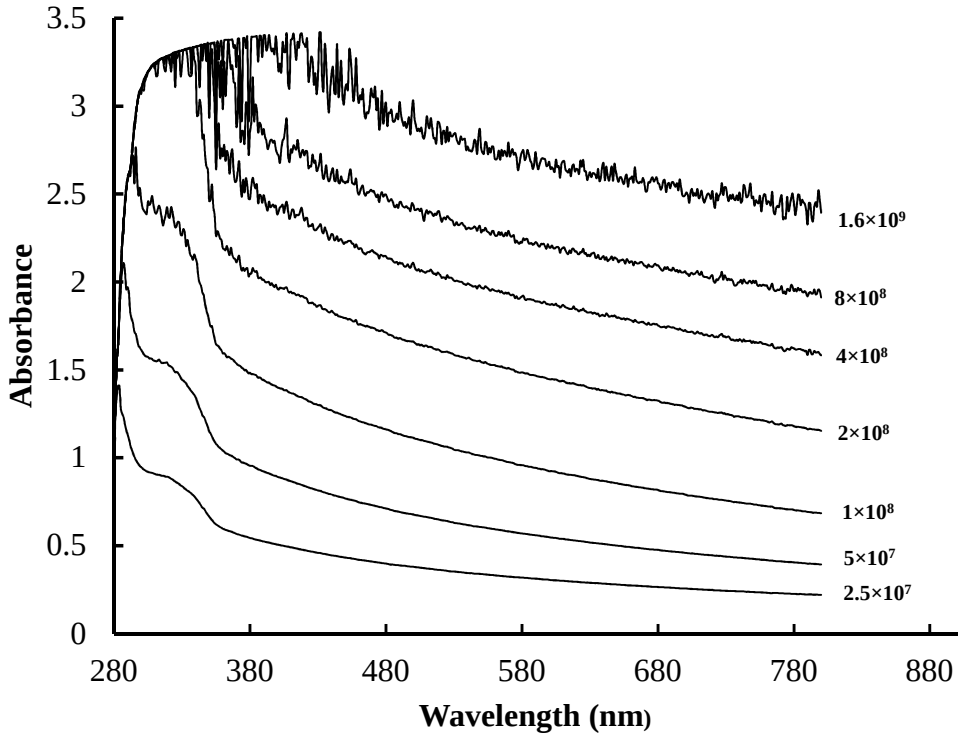

Fig. 1. Absorbance spectra of serially diluted *Ostrea angasi* sperm measured at the wavelengths from 280 to 800 nm.

The absorbance peaked at certain wavelength in each sperm concentration and reduced gradually with the increase in wavelengths. The wavelengths of 350, 450, 500 and 600 nm were primarily selected to determine the wavelength most suitable for sperm concentration estimation in *O. angasi*. The regression equations based on sperm concentration and absorbance at each wavelength were $y = 1\times10^{-8} x + 0.163$ (350 nm), $y = 8\times10^{-9} x + 0.146$ (450 nm), $y = 8\times10^{-9} x + 0.150$ (500 nm) and $y = 7\times10^{-9} x + 0.118$ (600 nm), respectively (Fig. 2). All the four wavelengths produced SLR equations but the best fitted regression model was derived at 350 nm ($r^2 = 0.996$).

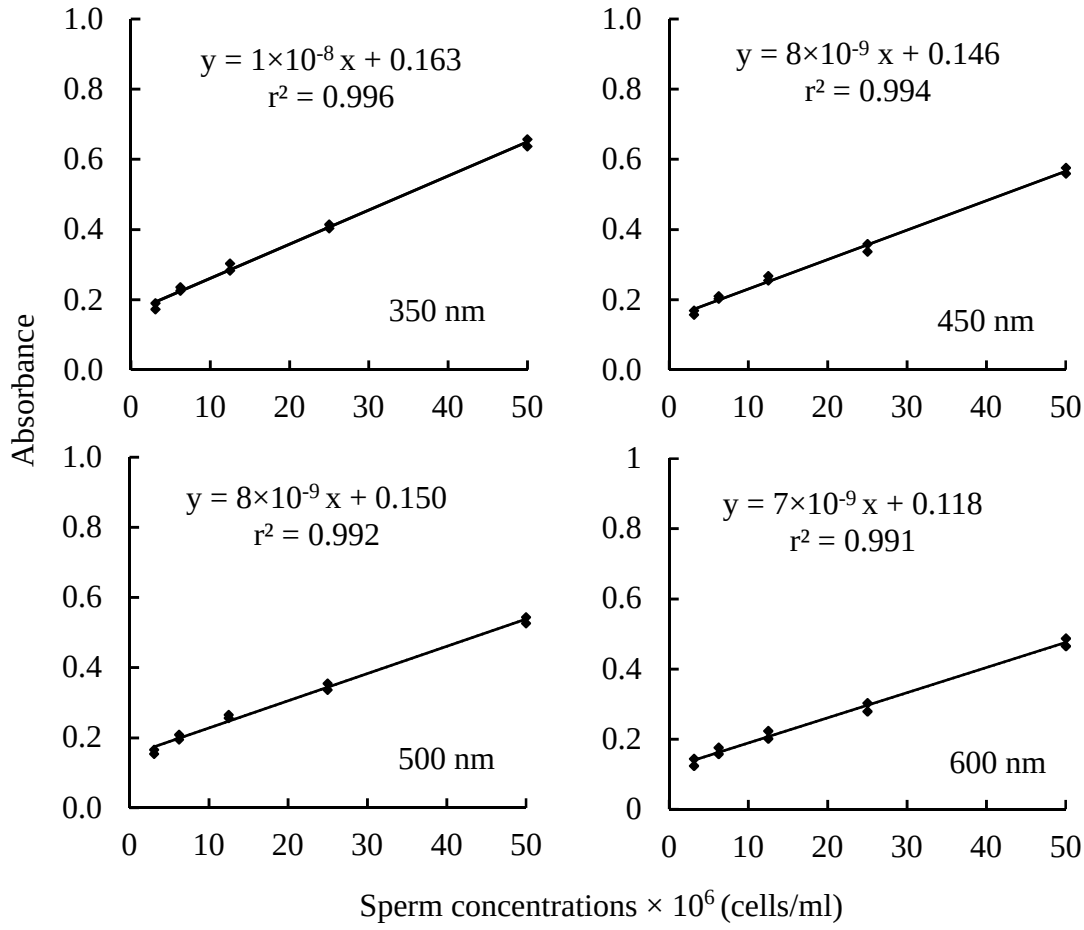

Fig. 2. Standard curves generated by the spectrophotometric technique using *Ostrea angasi* sperm at different wavelengths (350 nm, 450 nm, 500 nm and 600 nm).

5.4.2 Validation of the regression line

The sperm concentrations read from the regression line ($y = 1\times10^{-8} x + 0.163$) at 350 nm wavelength were validated by the haemacytometer method. The difference in sperm counts between the haemacytometer method and those calculated by the regression line was not significant ($P = 0.985$).

5.4.3 Repeatability of the spectrophotometric technique

There was no significant difference in sperm count between the haemacytometer method and the spectrophotometric technique in sample group 1 ($P = 0.921$), group 2 (P

= 0.992) and group 3 (P = 0.802), respectively.

5.5 Discussion

The use of light absorbance to measure concentration of suspended particles is a common practice in life science. In this study, the spectrophotometric technique was developed for estimating sperm concentrations in the spermcasting oyster *O. angasi*.

The determination of absorbance spectra at different concentrations is the first step in sperm quantification using the spectrophotometric technique. The spectra were generated by plotting the absorbance from 280 to 800 nm at a 1-nm interval to identify the wavelength of maximum sensitivity. In the spectra, a peak of absorbance indicates the most sensitive wavelength, and is recommended for spectrophotometric measurement (Harris, 1987). The absorbance gradually decreases with increasing wavelengths from the point of the most sensitive value. In this study, a peak of absorbance existed around 350 nm, and the wavelength of highest sensitivity at 350 nm or above would be most suitable for spectrophotometric measurement.

In general, sperm concentration and absorbance are linearly correlated but if the samples are too diluted and the absorbance was less than 0.1, the relation between absorbance and sperm concentration becomes curvilinear (Dong et al., 2005). When the absorbance is below 0.1, the polynomial regression model can be used for calibration. However, it would be better to exclude the data from over diluted samples because the linear model is more robust than the polynomial model for estimating sperm concentration. On the other hand, all the sperm do not absorb light properly if the samples are too dense due to the shadow effect among sperm. Consequently, absorbance above 2 represents less than 1% transmittance of the incident light (Poole and Kalnenieks, 2000). The absorbance above 1 represents 20% transmittance whereas

absorbance below 0.1 represents 10% transmittance. Absorbance values from 0.1 to 1.0 represent 70% transmittance and this absorbance range is recommended in spectrophotometric assays (Harris, 1987) which is also applied in this study.

In the photometric technique, light absorbance is proportional to the density of suspended particles. The timing between sample loading and absorbance reading is critical because sperm can sink to the bottom of the well in a few seconds. Stripping is the most common method for sperm collection in oysters but relics of gonad and somatic tissues might be present in the sperm suspension leading to a bias in absorbance measurement. The oyster gonad is anatomically mingled with digestive tissues, and contamination with gut contents would also affect calibration. The particle size, shape and homogeneity also affect absorbance. Therefore, sperm handling is critically important because a difference in the procedure can affect repeatability of the estimation.

The wavelengths for developing regression lines differ among previous studies. In this study, four wavelengths in the range of visible light 350, 450, 500 and 600 nm were compared to select an appropriate wavelength based on the best fit in the regression model. Sperm concentration can be estimated at any wavelength if a good fit in the regression line exists. The wavelength generating the best fit regression model differs in among species. For example, 400 nm was used in Atlantic croaker and small bodied fishes (Tan et al., 2010; Leclercq et al., 2012), 320 in blue mussels (Miguel and Beaumont, 2008), 581 nm in Pacific oysters (Dong et al., 2005) and 350 nm in the Australian flat oysters (present study). These differences suggest that the sperm absorbance spectra are species-specific and thus the regression model for sperm concentration estimation needs to be developed for each species.

Although the accuracy of haemacytometer count in this study was confirmed by immobilizing sperm with microwave, the addition of Lugol's iodine in the sperm

solution during haemacytometer count may change the sperm concentration. In future practice, the dilution factor of using Lugol's iodine should be considered. If necessary, Lugol's iodine should be added to sperm prior to the preparation of sperm solution and the volume of Lugol's solution added should be counted for calculating sperm concentration. The accuracy of spectrophotometric technique developed in this study was validated by the haemacytometer method. No significant difference in the sperm count was found between these two methods. The precision of spectrophotometric technique was further evaluated with sperm from different groups of individuals. Again no significant difference in sperm count was found, indicating the high repeatability of the spectrophotometric technique.

The spectrophotometry was established as an efficient and reliable technique for estimation of sperm concentrations in *O. angasi*. This technique measures absorbance of a sample within 10 sec, and is able to measure absorbance of at least 30 samples at a time (with three replicates in each sample) using a 96-well plate, whereas the haemacytometer method takes 10-15 min to count sperm of a single sample. It should be noted that the regression line generated in this study might not be applicable to other oyster species as the sperm-density and absorbance relationship might be species specific. This study would improve our capacity and efficiency in determining sperm concentration in *O. angasi* to facilitate the development of artificial breeding and other techniques such as cryopreservation.

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Chapter 6: Sperm cryopreservation in the spermcasting Australian flat oyster *Ostrea angasi* by a programmable freezing method

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6.1 Abstract

Cryopreservation offers long-term storage of gametes without constraint from seasonal gamete maturation, provides opportunities to improve the efficiency of breeding and genetic programs, and protects endangered species from extinction due to epidemic diseases and natural disasters. In this study, a protocol for cryopreserving sperm of the spermcasting Australian flat oyster *Ostrea angasi* was developed by optimizing key factors influencing the quality of cryopreserved sperm. Dimethyl sulfoxide (DMSO) was non-toxic to sperm within the concentration and duration assessed in the toxicity experiment whereas 10% methanol or a higher concentration was toxic to sperm from the exposure duration of 30 min onwards. DMSO produced higher post-thaw sperm motility of 21.6 % ± 2.7 % among the treatments with a single cryoprotectant. The inclusion of trehalose with DMSO increased the post-thaw sperm motility up to 33.3 ± 1.4 %. Sperm equilibrated for 30 min produced post-thaw motility of 32.2 ± 2.3 % and plasma membrane integrity (PMI) of 38.6 ± 2.0 % that were higher than those produced for 10 or 50 min. Higher post-thaw sperm motility of 37.7 ± 1.7 % and PMI of 42.8 ± 1.3 % PMI was produced at -3 ^{o}C/min than at -7 ^{o}C/min freezing. Sperm packaged in 0.5 ml straws had a higher post-thaw motility and PMI than those packaged in 0.25 ml straws. In this study, 44.4% post-thaw sperm motility and 49.2% PMI were achieved when sperm were equilibrated in 10% DMSO + 0.45 M trehalose for 30 min, packaged in 0.5 ml straws, frozen at -3 ^{o}C/min from 4 ^{o}C to -80 ^{o}C, and thawed at 40 ^{o}C for 8 sec. The availability of viable cryopreserved sperm would open an option for future breeding and genetic improvement programs for the spermcasting Australian flat oyster.

Keywords: Sperm cryopreservation, flat oyster, *Ostrea angasi*, mollusc

6.2 Introduction

Cryopreservation is the most practical option for long-term storage of biological specimen. With the increasing importance of preserving genetic materials for future research, there is a strong need to develop a reliable cryopreservation protocol to ensure long-term storage of gametes (Stacey and Day, 2014). Ever since the revolutionary invention of the cryoprotective capacity of glycerol to improve the survival of post-thaw sperm in human (Polge et al., 1949), the cryopreservation technique has been applied to study cellular and developmental biology of many terrestrial and aquatic species such cow, horse, lamb and fish. The worldwide dairy cattle breeding programs are heavily dependent on cryopreservation for distribution of genetically improved sperm but application of cryopreserved sperm in aquatic species is limited to a laboratory scale (Tiersch et al., 2007). Recent reviews have demonstrated the applicability of cryopreserved sperm in aquaculture if quality controls and biosecurity measures are ensured (Hassan et al., 2015; Martínez-Páramo et al., 2016). Cryopreservation protocols have been developed for over 200 aquatic species (Tiersch, 2000) since the successful cryopreservation of the Pacific herring sperm (Blaxter, 1953). Compared to mammals and fishes, cryopreservation research on marine molluscs started relatively late (Liu et al., 2015) but considerable progress has been made in commercially important species such as oysters (Dong et al., 2005a; Yang et al., 2012), pearl oysters (Kawamoto et al., 2007), mussels (Di Matteo et al., 2009; Smith et al., 2012), scallops (Espinoza et al., 2010), clams (Dupré et al., 2011) and abalone (Liu et al., 2014a).

Oysters are classified into broadcasting and spermcasting species depending on where the fertilization and early larvae development occur. The broadcasting species release gametes in seawater where the fertilization and larval development take place. The spermcasting species, on the other hand, produce spermatozeugmata which are inhaled by the spawning females to fertilize eggs (O'Foighil, 1989; Hassan et al., 2016).

The Australian flat oyster *Ostrea angasi*, a spermcasting marine mollusc, perform both fertilization and embryo incubation inside the shells for two to three weeks before releasing larvae in seawater (O'Sullivan, 1980). Even though over 50 research papers have been published on oyster sperm cryopreservation (Hassan et al., 2015) including high throughput sample processing for large scale application (Adams et al., 2004; Yang et al., 2012), past research has almost entirely focused on broadcasting oysters. Sperm cryopreservation research in spermcasting oysters is at infancy, with established protocols for only the European flat oyster *Ostrea edulis* (Vitiello et al., 2011; Horváth et al., 2012) and the Chilean flat oyster *Tiostrea chilensis* (Adams et al., 2013).

The structural and functional integrity of cryopreserved sperm can be compromised by cryoprotectant toxicity, non-equilibrium between extracellular and intracellular solutes, and intracellular ice formation during freezing and thawing (Watson, 2000). The intracellular ice formation can be prevented by adding cryoprotectants, but depending on the concentration and exposure time, cryoprotectants might be toxic to sperm (Fahy, 1986). Therefore, optimizing cryoprotectant concentration and exposure time is critical to minimize toxicity and maximize protection for gametes. Identifying suitable freezing rate is another critical factor in cryoprervation because slow freezing prevents intracellular ice formation whereas fast freezing minimizes toxic effect at a higher solute concentration. Intracellular ice and toxicity of solute both contribute to cell damage, so the freezing rate should be slow enough to minimize ice formation and fast enough to minimize the solute effect. In addition, the sample volume can also affect the rate of heat transfer during freezing and thawing (Xu et al., 2010). Therefore, sample volume should be optimized to ensure reproducibility of a cryopreservation protocol.

The spermcasting oyster *O. angasi* used to be an important species for fishery in a few Australian states but this species was over exploited in the late 18th and early 19th

centuries (Nell, 2001; Alleway and Connell, 2015). Even though harvesting has stopped for decades, the natural population of *O. angasi* has not been recovered. Recently, the aquaculture production of *O. angasi* has increased gradually due to (a) an increasing interest to diversify oyster aquaculture and (b) a high consumer demand on seafood markets (Heasman et al., 2004). The development of selective breeding programs would further facilitate the sustainable aquaculture development in this species. Pedigreed families can be established by using cryopreserved sperm which allow greater control over parental crosses in breeding programs. In addition, cryopreserved sperm allow the production of self-fertilized inbred stocks to facilitate exploitation of heterotic vigour. This study aimed to develop a cryopreservation protocol of *O. angasi* to facilitate future breeding and genetic improvement programs. A programmable freezing method was developed and the factors affecting sperm quality during cryopreservation were optimized using sperm motility and plasma membrane integrity as assessment indicators.

6.3 Materials and methods

6.3.1 The oysters

Two-year old flat oysters (76.2 ± 4.7 mm in shell length and 70.3 ± 12.1 g in total weight) were provided by the Pristine Oyster Farm in Coffin Bay, South Australia within the natural spawning season from July to October, 2014 and transported to South Australian Research and Development Institute in a chilled Styrofoam box. On arrival, the oysters were cleaned with brush and placed in a rectangular tank supplied with flow-through seawater and aeration. The water temperature was maintained at 20 ± 0.5 °C and oysters were fed a microalgal mixture of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans*.

6.3.2 Sperm collection

After opening the shells, sperm were collected by stripping the gonad using a 3.5 ml Pasteur pipette. To separate sperm from gonad tissues and spermatozeugmata, the sperm suspension was filtered through a 45 µm screen, which was fitted between two 1 mL pipette tips with the inner tip cut at 3 cm from the narrow end. The sperm samples were filtered by a downward push with a pipette. Sperm concentration was estimated with the spectrophotometric method and standardized to 1×10^9 cells/ml by diluting with filtered seawater in all the experiments (Hassan et al., 2017). Sperm pooled from 3-5 males were used in each experiment.

6.3.3 Sperm quality assessment

Sperm quality was assessed by estimating the percentage of motile sperm and plasma membrane integrity (PMI). Sperm motility was video-recorded using an Olympus BX50 microscope and blind assessed by two observers. An aliquot of 2 µl sperm was placed on a glass slide and 20 µl filtered seawater was added during sperm motility assessment. The motility percentage from two observers was averaged as sperm motility of the subsample. Three subsamples were collected in each replicate. Sperm moving forward were considered motile whereas those vibrating in place or not moving at all were considered non-motile. The fresh sperm with at least 50 percent motility were used in the experiments.

The sperm PMI was assessed by using the molecular probes LIVE/DEAD® sperm viability kit (L-7011). During PMI evaluation, cryopreserved sperm concentration was adjusted to 1×10^8 cells/ml with filtered seawater. The dye was thawed at room temperature after being taken out of the freezer and the container was tapped 8-10 times by a finger to ensure homogeneity. An aliquot of 1 µl SYBR 14 was added to 100 µl sperm for 10 min in dark, and then 3 µl of propidium iodide for further

10 min in dark. Aliquots of 10 µl stained sperm were observed on an Olympus BX60 fluorescence microscope. At least 100 sperm cells were counted in each replicate. Sperm emitted green and red fluorescence were considered live and dead, respectively.

6.3.4 Experiments

6.3.4.1 Cryoprotectant toxicity during storage on ice

Permeable cryoprotectants, dimethyl sulfoxide (DMSO) and methanol were used to test cryoprotectant toxicity during sperm equilibration. Both were used at 5, 10 and 15% in the final sperm suspension. Sperm motility was evaluated as a measure of cryoprotectant toxicity after 10, 30 and 50 min exposure.

6.3.4.2 Cryoprotectant selection

In the initial step, the effect of single cryoprotectant on post-thaw sperm motility was evaluated. Sperm were equilibrated with 5, 10 and 15% DMSO or methanol, and cryopreserved. The following cryopreservation protocol was used in both steps. Sperm were equilibrated with cryoprotectants for 30 min, packaged in 0.25 ml French straws, frozen at -7° C/min using a computer controlled freezer (FREEZE CONTROL® CL-863) from 4 ^{o}C to -80 ^{o}C, immediately plunged in liquid nitrogen, and finally stored in the liquid nitrogen dewar. The frozen sperm were thawed at 40 $^{\circ}$C water bath for 6 sec prior to sperm motility evaluation.

In the final step, the combined effect of permeable and non-permeable cryoprotectants on post-thaw sperm motility was evaluated, with the former being 5, 10 and 15% DMSO and the latter 0.45 M trehalose or 0.3 M glucose (final concentrations).

6.3.4.3 Effect of equilibration duration on post-thaw sperm motility and PMI

Post-thaw motility and PMI of sperm equilibrated for 10, 30 and 50 min in 10% DMSO + 0.45 M trehalose were evaluated. The freezing and thawing protocols described in Experiment 6.3.4.2 were used in this experiment.

6.3.4.4 Effects of freezing rate on post-thaw sperm motility and PMI

The effects of freezing rate on post-thaw sperm motility and PMI were evaluated with -3 °C/min and -7 °C/min freezing rates. Sperm were equilibrated in 10% DMSO + 0.45 M trehalose for 30 min, packaged in 0.25 ml straws, and thawed at a 40 °C water bath for 6 sec.

6.3.4.5 Effects of sample volume on post-thaw sperm motility and PMI

The effects of sample volume on post-thaw motility and PMI were evaluated by packaging sperm in 0.25 ml and 0.5 ml straws. Sperm were equilibrated in 10% DMSO + 0.45 M trehalose for 30 min and frozen at -3 °C/min. The 0.25 ml and 0.5 ml straws were thawed for 6 sec and 8 sec, respectively at a 40 °C water bath.

6.3.4.6 Cold storage of sperm

Sperm were stored in a refrigerator at 4 ºC to evaluate the effect of cold storage on sperm motility. Sperm were suspended in filtered seawater in the control group. In the three treatment groups, sperm were separately equilibrated with the final cryoprotectant concentrations of 5% DMSO, 5% DMSO + 0.45 M trehalose, and 5% DMSO + 0.3 M glucose. Subsamples were warmed at 20 ºC for 20 min prior to motility assessment. Sperm motility was evaluated at a daily interval for up to seven days.

6.3.5 Statistical analysis

For statistical analysis, fresh sperm motility was set to 100 percent while post-equilibration and post-thaw motilities were calculated as a relative percentage of fresh sperm motility to standardize motility in different treatments. However, the actual motility percentages are presented in the results. The relative sperm motilities were then arcsine transformed prior to statistical analysis. Depending on the number of factors involved, treatment effects on sperm motility and PMI in experiments 6.3.4.1, 6.3.4.2, 6.3.4.3, and 6.3.4.6 were analysed with one-factor or two-factor analysis of variance (ANOVA). A repeated measures design was applied when data were collected at intervals. In experiments 6.3.4.4 and 6.3.4.5, treatment effects on sperm motility and PMI were analysed with independent sample t-test. Data were expressed as mean ± SE. The differences in mean were examined by Duncan's multiple range test (DMRT) and considered significant at $P < 0.05$. The data were analyzed with SPSS version 20.0 (IBM Corporation, Armonk, NY, USA).

6.4 Results

6.4.1 Cryoprotectant toxicity

Based on sperm motility, up to 15% DMSO concentration and 50 min exposure was non-toxic to sperm. The 5% methanol was non-toxic whereas sperm motility decreased significantly after 30 min exposure to 10% or a higher concentration of methanol ($P < 0.05$). The toxicity of methanol increased with increasing its concentrations and equilibration durations (Table 1).

Table 1: Sperm motility (%) after exposure to 5, 10 and 15% dimethyl sulfoxide (DMSO) and methanol for 10, 30 and 50 min (n = 5). Different superscript letters represent significant differences in sperm motility.

Cryoprotectant type	Cryoprotectant concentration (%)	Motility (%) at different equilibration durations (min)		
		10	30	50
DMSO	5	58.3 ± 1.5 [a]	57.9 ± 1.6 [a]	58.7 ± 1.7 [a]
	10	59.1 ± 1.9 [a]	56.7 ± 1.7 [a]	56.7 ± 1.9 [a]
	15	58.7 ± 1.7 [a]	58.0 ± 1.7 [a]	58.0 ± 2.0 [a]
Methanol	5	58.0 ± 1.4 [a]	54.7 ± 1.3 [a]	56.7 ± 1.6 [a]
	10	59.3 ± 1.5 [a]	52.0 ± 1.1 [ab]	46.7 ± 1.6 [b]
	15	57.3 ± 1.5 [a]	48.0 ± 1.1 [b]	43.3 ± 2.3 [b]

6.4.2 Cryoprotectant selection

Sperm cryopreserved with DMSO had significantly higher post-thaw motility than those cryopreserved with methanol ($P < 0.05$). Among the DMSO concentrations evaluated, 10% DMSO produced the highest post-thaw sperm motility, which was similar to 15% DMSO but significantly higher than that produced with 5% DMSO ($P < 0.05$; Fig. 1).

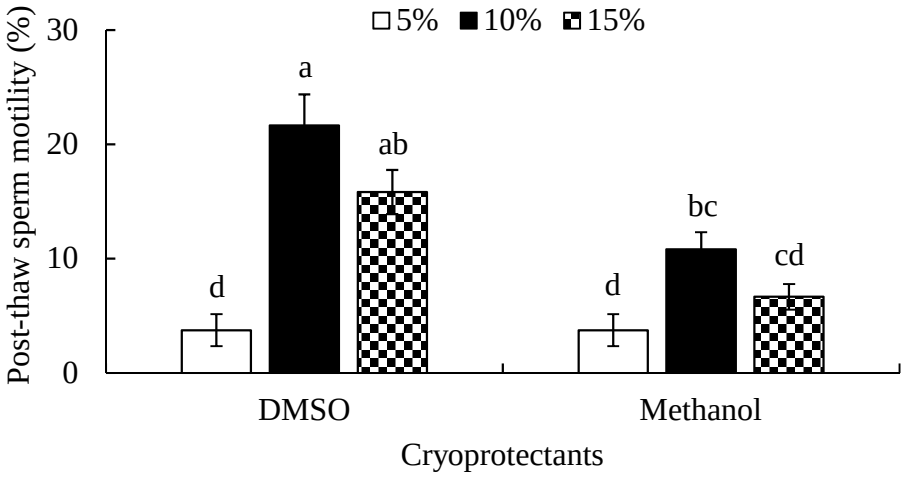

Fig. 1: Post-thaw motility (%) of sperm cryopreserved with different concentrations of dimethyl sulfoxide (DMSO) and methanol. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of four replicates. Different letters represent significant differences in sperm motility.

The inclusion of trehalose and glucose in DMSO has improved the post-thaw sperm motility in all DMSO concentrations assessed (Figs. 1 and 2). Sperm cryopreserved with 10% DMSO + 0.45 M trehalose had significantly higher post-thaw motility than those cryopreserved with other cryoprotectants ($P < 0.05$; Fig. 2).

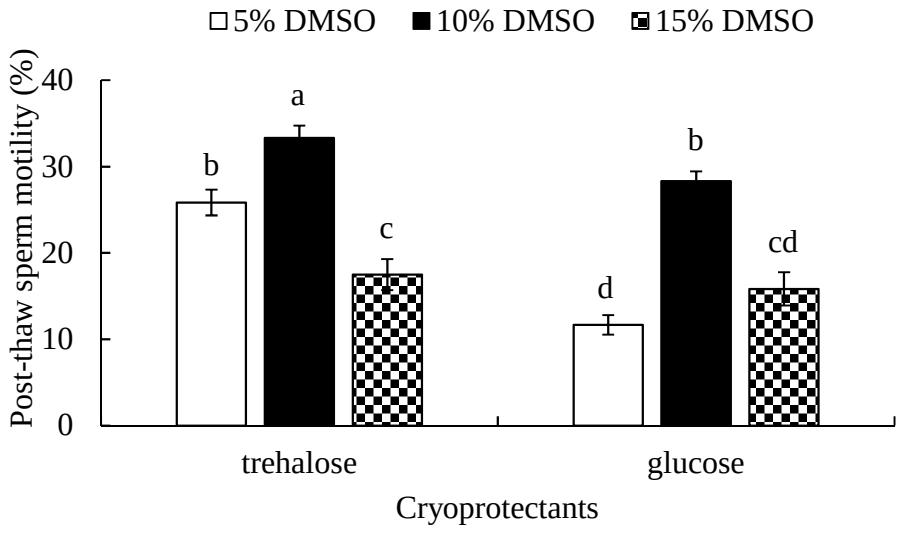

Fig. 2: Post-thaw motility (%) of sperm cryopreserved with different concentrations of dimethyl sulfoxide (DMSO) + 0.45 M trehalose or 0.3 M glucose. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of four replicates. Different letters represent significant differences in sperm motility.

6.4.3 Equilibration duration

The highest post-thaw motility of 32.2% and PMI of 38.7% were achieved in this experiment by equilibrating sperm for 30 min. Sperm cryopreserved after 30 min equilibration had significantly higher post-thaw motility and PMI than those cryopreserved after 50 min and 10 min equilibration ($P < 0.05$; Fig. 3).

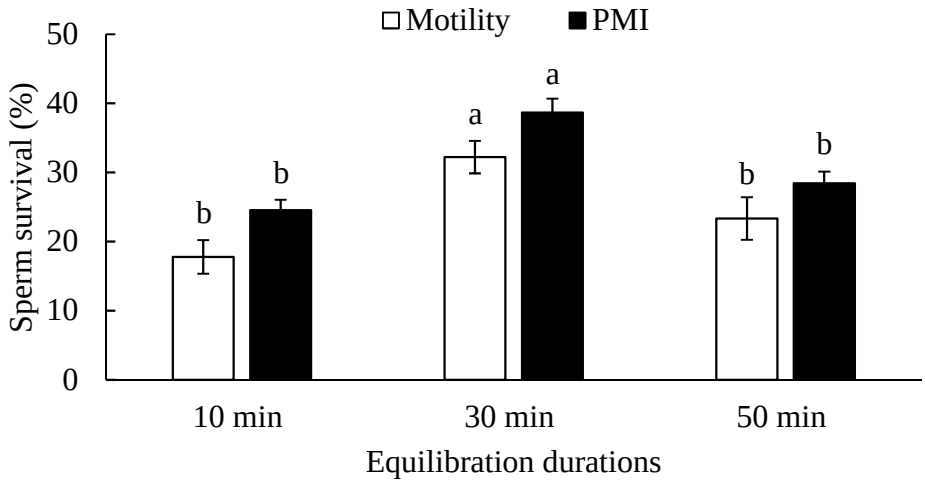

Fig. 3: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved after different equilibration durations in 10% dimethyl sulfoxide + 0.45 M trehalose. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of three replicates. Different letters represent significant effect of equilibration durations on sperm motility or PMI.

6.4.4 Effects of freezing rate on post-thaw sperm motility and PMI

The highest post-thaw motility of 37.7% and PMI of 42.8% were achieved in this experiment by freezing sperm at -3 ºC/min from 4 ºC to -80 ºC. Sperm frozen at -3 °C/min had significantly higher post-thaw motility and PMI than those frozen at -7 °C/min ($P < 0.05$; Fig. 4).

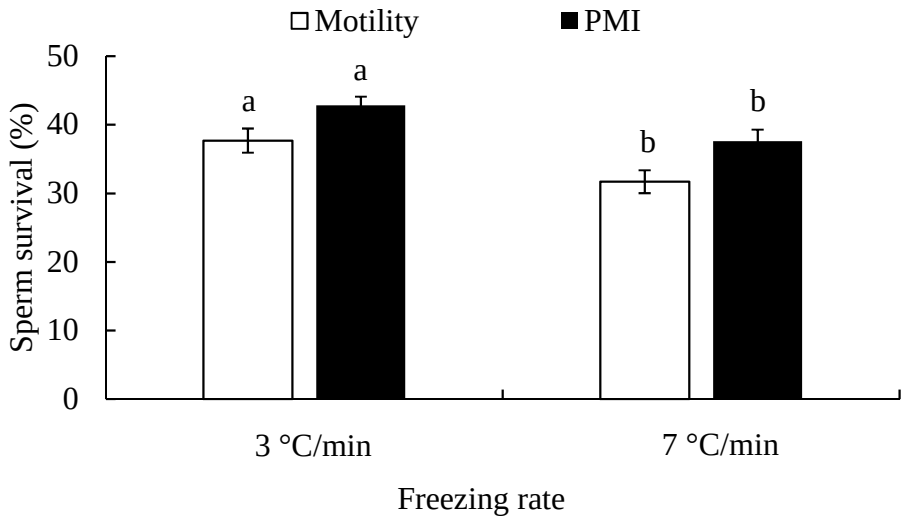

Fig. 4: Post-thaw motility and plasma membrane integrity (PMI) of sperm frozen at -3 °C/min and -7 °C/min, respectively. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of three replicates. Different letters represent significant effect of freezing rates on sperm motility or PMI.

6.4.5 Effects of sperm volume on post-thaw sperm motility and PMI

The highest post-thaw motility of 44.4% and PMI of 49.2% were achieved in this experiment by packaging sperm in 0.5 ml straws. Sperm packaged in 0.5 ml straws during cryopreservation had significantly higher post-thaw motility and PMI than those packaged in 0.25 ml straws ($P < 0.05$; Fig. 5).

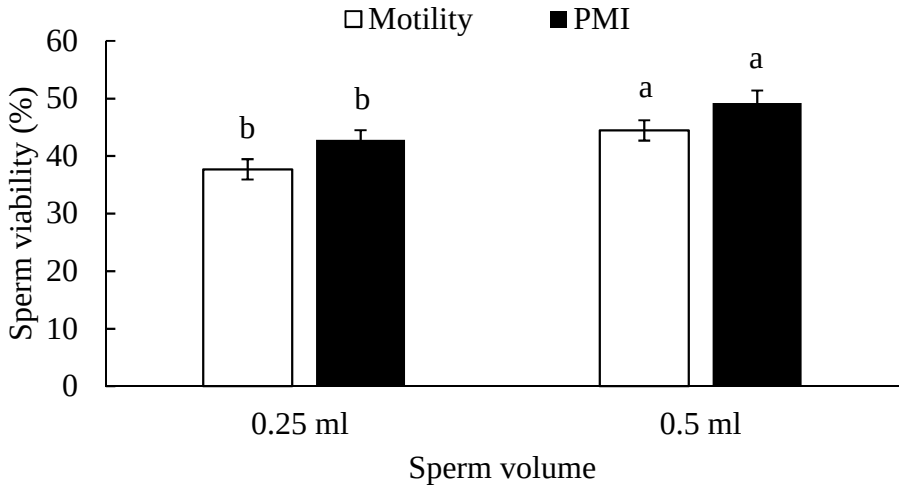

Fig. 5: Post-thaw motility and plasma membrane integrity (PMI) of sperm cryopreserved in 0.25 ml and 0.5 ml straws. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of three replicates. Different letters represent significant effect of straw volumes on sperm motility and PMI.

6.4.6 Sperm motility during cold storage

During cold storage, sperm motility decreased with increasing storage duration. Motile sperm existed in the untreated samples for five days but the inclusion of 5% DMSO + 0.45 M trehalose extended sperm motility duration to day 7 (Fig. 6). The inclusion of 5% DMSO or 5% DMSO + 0.45 M trehalose significantly improved sperm motility compared with those stored in filtered seawater ($P < 0.05$). However, the inclusion of 5% DMSO + 0.3 M glucose did not improve sperm motility ($P = 0.368$).

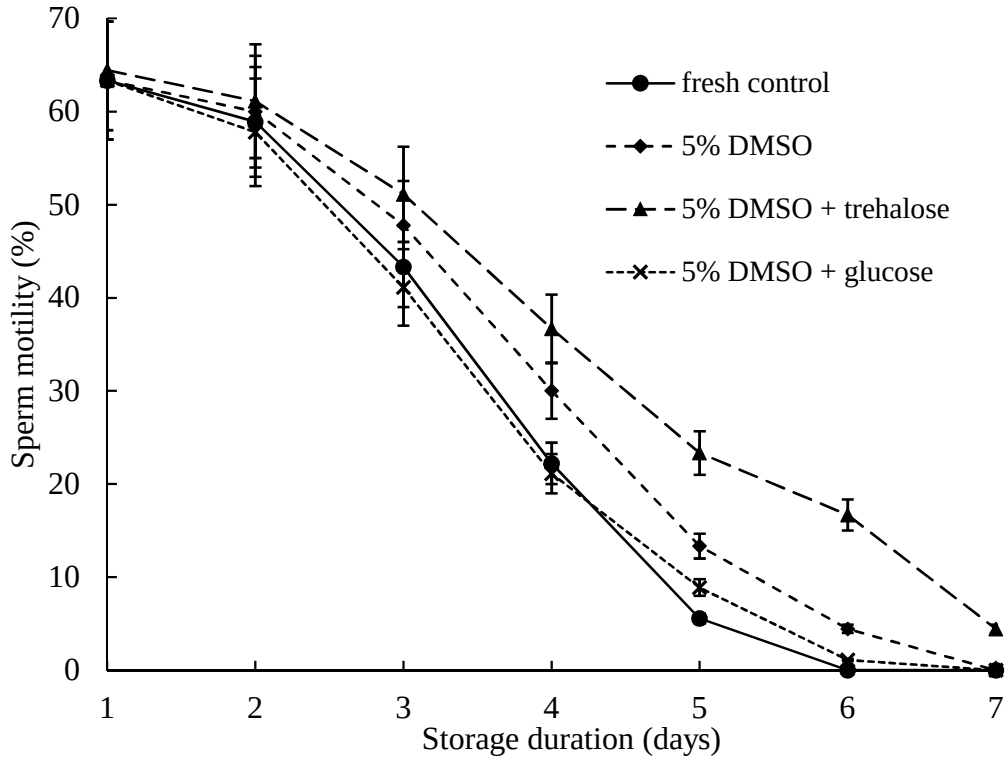

Fig. 6: Motility (%) of sperm stored in the refrigerator at different durations. Sperm were suspended in 5% dimethyl sulfoxide (DMSO), 5% DMSO + 0.45 M trehalose or 5% DMSO + 0.3 M glucose. (n = 3).

6.5 Discussion

The protocol of sperm cryopreservation in the spermcasting Australian flat oyster was developed by optimizing cryoprotectant types, equilibration duration, freezing rate and sperm volume. Although the capacity of post-thaw sperm to fertilize eggs is the key criterion for assessing ultimate sperm quality, a reliable protocol for artificial fertilization is not available in this species. Therefore, sperm quality was assessed by motility and PMI evaluation in this study. In general, sperm motility and PMI have a strong correlation with fertility (Au et al., 2002; Moskovtsev et al., 2005; Fabbrocini et al., 2016).

Cryoprotectant is an essential component except for cryoprotectant free vitrification in cryopreservation. The permeable cryoprotectants provide better protection but may exert a toxic effect on sperm (Arakawa et al., 1990). Based on sperm motility, up to 15% DMSO for 30 min equilibration was non-toxic but 10% methanol and higher concentrations were toxic when sperm were equilibrated for 30 min or longer. Sperm of different species have different toxicity response to a cryoprotectant. For example, methanol is non-toxic but DMSO is toxic to the European flat oyster sperm (Vitiello et al., 2011), whereas DMSO was non-toxic but methanol was toxic to the Australian flat oyster sperm. In teleost, sperm have been found immotile within a few minutes of equilibration with 15% DMSO (Nahiduzzaman et al., 2011), whereas this concentration was within the toxic threshold in the Australian flat oyster sperm.

High permeability and low toxicity are the most desirable characteristics for cryoprotectants to prevent intracellular ice formation or shrinkage of cells during freezing and thawing (Fahy, 1986). DMSO permeates into cells faster therefore cells quickly reach equilibrium with the extracellular solutes (MacGregor, 1967). In sperm cryopreservation of most the marine mollusc species, 5-20% DMSO was used as a suitable cryoprotectant as reviewed by Liu et al. (2015). In this study, both 10% and 15% DMSO produced significantly higher post-thaw motility than 5% DMSO. Even though 15% DMSO was not significantly different from 10% DSMO, a lower post-thaw sperm motility with the former suggests that 15% DMSO might be the threshold level for a solute effect in this cryoprotectant. The lower post-thaw motility of sperm cryopreserved with 5% DMSO suggests that this concentration is insufficient to protect sperm from cellular injury. The methanol, another permeable cryoprotectant evaluated in this study, showed higher toxicity and produced low post-thaw sperm motility than DMSO.

The addition of non-permeable cryoprotectants improves sperm motility during cryopreservation (Liu et al., 2014b). The functional differences between permeable and non-permeable cryoprotectants are that the former enters the cell by replacing water molecule (Harvey and Ashwood-Smith, 1982) while the latter tends to stabilize cell membrane from outside (Strauss et al., 1986), though this generalization may vary among species. The cocktail of permeable and non-permeable cryoprotectants improve sperm viability by improving cell membrane stability and cohesiveness (De Leeuw et al., 1993). In addition, the non-permeable cryoprotectants change the properties of extracellular solutions and prevent ice crystallization. Trehalose, for example, stops eutectic freezing by trapping salts and prevents mechanical damage to sperm (Woelders et al., 1997). Trehalose, glycine, glucose, sucrose and egg yolk are the commonly used non-permeating cryoprotectants for sperm cryopreservation in marine molluscs (Salinas-Flores et al., 2005; Adams et al., 2008; Liu et al., 2015). In this study, the post-thaw sperm motility was improved by the inclusion of 0.45 M trehalose or 0.3 M glucose in 10% DMSO. This result agrees with the findings in other bivalves such as the Pacific oyster (Adams et al., 2004), Chilean flat oyster (Adams et al., 2013), pearl oyster (Acosta-Salmón et al., 2007) and greenlip abalone (Liu et al., 2014b).

Equilibration is the exposure of sperm to a cryoprotectant for balancing solute influx and water outflow. Oyster sperm can be cryopreserved at 5 to 60 min equilibration depending on the cryoprotectant type and concentration (Hassan et al., 2015). While shorter equilibration might result in insufficient internal solute to protect sperm from intracellular ice formation and/or shrinkage, the longer equilibration might exert solute and toxic effect on sperm. Cryoprotectant concentration has a mutual effect on the equilibration duration and a higher cryoprotectant concentration requires a shorter equilibration duration or vice-versa. In this study, relatively higher post-thaw sperm motility and PMI were found at 30 min equilibration than at 10 and 50 min

equilibration with 10% DMSO + 0.45 M. This means that 10 min equilibration might result in insufficient internal solutes but 50 min equilibration could exert a solute effect on sperm. Similar to this study, 30 min cryoprotectant equilibration is optimal for cryopreservation in other oyster species (Ieropoli et al., 2004; Dong et al., 2005a; Horváth et al., 2012).

Freezing is the most critical step in cryopreservation because cell damage mostly occurs from 0 to -40 °C (Leung, 1991). Due to a number of interacting factors and species specificity in sperm cryopreservation, independent studies have found optimum freezing rates from -1 °C/min to -50 °C/min as reviewed by Hassan et al. (2015). In this study, -3 °C/min freezing produced significantly higher post-thaw sperm motility and PMI than -7 °C/min. In the spermcasting European flat oyster, significant differences were found among the freezing rates of -1, -3, -7, -10 °C/min, and the highest post-thaw sperm motility was achieved at -3 °C/min (Vitiello et al., 2011). Results from these two spermcasting species indicate that sperm of spermcasting oyster species might be more sensitive than other species.

As the sample volume for cryopreservation is a factor influencing sperm quality, the reported findings on sperm cryopreservation sometimes differ among species (Cabrita et al., 2001; Aoki et al., 2007). A larger vial is usually suitable for sperm cryopreservation at a hatchery scale but the heterogeneity of temperature inside a large vial during freezing and thawing may affect sperm quality. Although sperm cryopreservation is successful in larger vials, 0.25 ml and 0.5 ml straws are used in most studies (Hassan et al., 2015; Liu et al., 2015). In this study, the post-thaw sperm motility and PMI were higher in a 0.5 ml straw than in a 0.25 ml straw. Sperm packaged in the 0.5 ml straw produced higher sperm viability than in 0.25 and 5 ml straws in teleosts and other vertebrates (Cabrita et al., 2001; Buranaamnuay et al., 2009). Sperm packaged in 0.25 ml straws, on the other hand, produced higher motility than those in 1-2 ml

straws in the Japanese pearl oyster (Aoki et al., 2007). In contrary, the impact of straw size on post-thaw motility of sperm in Pacific oyster was not significant (Dong et al., 2005b).

Sperm storage in a refrigerator is an option when sperm cannot be cryopreserved immediately after collection as liquid nitrogen may not be accessible in some situations, especially in a remote area. Sperm suspended in seawater were motile for five days during refrigerator storage, whereas the inclusion of 5% DMSO + 0.45 M trehalose increased storage duration up to seven days. The duration of sperm survival after cold storage in the Australian flat oyster is similar to that of the Pacific oyster (Vanderhorst et al., 1985). It seems that cryoprotectants could establish equilibrium between sperm and the extracellular solutes during cold storage and the inclusion of a cryoprotectant could increase the duration of sperm storage without compromising sperm quality. This result indicates that DMSO and trehalose are useful cryoprotectants for short-term sperm storage in the Australian flat oyster, which is similar to the results of sperm cold storage in teleost species (Linhart et al., 1995; Hassan et al., 2013).

In summary, 44.4% post-thaw motility and 49.2% PMI were achieved by applying the protocol developed in this study. This protocol required that sperm are equilibrated in 10% DMSO + 0.45 M trehalose for 30 min, packaged in 0.5 ml straws, frozen at -3 ºC/min from 4 ºC to -80 ºC, and thawed at 40 ºC for 8 sec. Further research, however, is needed to focus on the development of strategies for the application of cryopreserved sperm to facilitate the development of artificial fertilization in spermcasting oyster species.

6.6 Acknowledgements

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Chapter 7: Improvement of post-thaw sperm survivals using liquid nitrogen vapour technique in a spermcasting oyster *Ostrea angasi*

This chapter has been submitted to Cryobiology as:

Hassan, M. M., Li, X. & Qin, J. G. Improvement of post-thaw sperm survivals using liquid nitrogen vapour technique in a spermcasting oyster *Ostrea angasi*.

7.1 Abstract

Low survival of cryopreserved sperm impedes the application of cryopreservation technique in spermcasting oyster species. This study developed a simple method of liquid nitrogen vapor freezing to improve post-thaw sperm survival in the spermcasting oyster *Ostrea angasi*. The results indicate that the permeable cryoprotectants, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) were non-toxic to sperm up to 20% concentration and 90 min exposure whereas methanol at 10% or higher was toxic to sperm for any exposure over 30 min. Among the treatments with permeable cryoprotectants, 15% EG produced the highest post-thaw sperm motility. Sperm motility was further improved by the addition of non-permeable cryoprotectants (trehalose and glucose), with 15% EG + 0.2 M trehalose resulting in the highest post-thaw sperm motility among all the combinations evaluated. The durations of 20, 30 and 60 min equilibrations produced a higher post-thaw sperm motility and plasma membrane integrity (PMI) than 10 min. Higher post-thaw motility and PMI were achieved by freezing sperm at the 8 cm height from the liquid nitrogen surface than at the 2, 4, 6, 10 or 12 cm height. Holding sperm for 10 min in liquid nitrogen vapor produced higher post-thaw motility and PMI than for 2, 5 or 20 min. The cryopreservation protocol developed in this study could increase both post-thaw sperm motility and PMI by at least 15% higher than those using the programmable freezing method. The cryopreservation protocol developed in this study has improved the sperm quality in spermcasting oyster species.

Keywords: Cryopreservation, freezing, sperm survival, mollusc

7.2 Introduction

Cryopreservation is a promising method for storage of important genotypes in the application of aquaculture biotechnology. In the past 50 years, sperm cryopreservation research in marine invertebrates has made substantial progress in marine aquaculture (Moragas et al., 2012; Hassan et al., 2015) and marine environment health assessment (Fabbrocini et al., 2012). Among marine invertebrates, the edible oysters are most widely studied (Paredes, 2015), but 95% of the work has focused on broadcasting species (Hassan et al., 2015). The spermcasting species have unique spermatological characteristics as the sperm are released in clusters of spermatozeugma (Hassan et al., 2016). The motility of post-thaw sperm achieved in previous studies was low (Vitiello et al., 2011; Horváth et al., 2012) probably because the sperm of spermcast spawning species are more sensitive to the cryopreservation process. In a preliminary study in our lab (unpublished), only 44% post-thaw motility and 49% plasma membrane integrity (PMI) were achieved in the sperm of the Australian flat oyster *Ostrea angasi*. Therefore, sperm quality needs to be further improved to increase the efficiency of cryopreservation in future breeding and genetic improvement programs in spermcasting species.

Different freezing methods have been successfully used for cryopreservation such as programmable computer controlled freezing and liquid nitrogen vapor freezing. Even though the programmable freezing is a widely-used method for sperm cryopreservation, high sperm survival has achieved using the liquid nitrogen vapor method in many marine invertebrates such as 92% post-thaw fertilization in the Pacific oyster (Zhang et al., 2012), 95% fertilization in the green-lip abalone (Zhu et al., 2014), and 80% fertilization in the greenshell mussel (Smith et al., 2012), and 86% motility in the pearl oyster (Lyons et al., 2005). The liquid nitrogen vapour freezing method has also been suggested to establish a gamete cryobanking service to the Australian

aquaculture industry due to low initial investment and subsequent maintenance cost (Li, 2012). Freezing with liquid nitrogen vapor does not require expensive equipment and highly skilled personnel. As a large quantity of sperm can be cryopreserved within a short time in a single batch, this method is considered simple, less expensive and efficient. However, the application of liquid nitrogen vapor method to cryopreserve the Australian flat oyster sperm has not yet been evaluated.

The quality of sperm can be compromised by the cryopreservation process. However, optimization of the steps in cryopreservation can improve the ability of sperm to withstand damages from cryopreservation. Although cryoprotectants could minimize freezing and thawing injury, they may also be toxic to sperm at a high concentration. Therefore, the evaluation of the sperm tolerance to cryoprotectants should be the first step for cryoprotectant selections. The cryoprotectants consist of two types of chemicals: the permeable and non-permeable cryoprotectants. The former enters into the cell and bring an equilibrium between extracellular and intracellular solutes whereas the latter stabilizes the cell membrane and increase cell membrane cohesiveness (De Leeuw et al., 1993). Therefore, suspending sperm with both types of cryoprotectants may minimize the chance of intracellular ice formation and cell membrane shrinkage. Freezing is the most important step in a cryopreservation procedure because most sperm injuries occur within the temperature range of 0 °C - 40 °C due to the formation of intracellular ice. In liquid nitrogen vapor freezing method, the distance of the sample from the liquid nitrogen surface, holding duration of samples in liquid nitrogen vapor and straw size can all affect the application of the cryopreservation protocol and need to be optimised. This study aimed to develop a non-programmable freezing technique to improve the post-thaw sperm quality of the Australian flat oyster, including the optimization of cryoprotectant concentration, equilibration duration, sperm distance to liquid nitrogen surface, holding duration, and sperm volume.

7.3 Materials and methods

7.3.1 The oysters

Pristine Oyster Farm in Coffin Bay, South Australia provided the two years old flat oysters (76.8 ± 4.4 mm in shell length and 71.2 ± 11.8 g in total weight). The oysters were shipped to South Australian Research and Development Institute in a chilled Styrofoam box during September and November, 2014. The oysters were cleaned off debris and epifauna, and placed in tanks supplied with flow-through seawater and aeration. Mixed microalgae of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans* were supplied to the tank and water temperature was maintained at 20 ± 0.5 $^{\circ}$C.

7.3.2 Freezing apparatus

A Styrofoam box (39.0 × 24.5 × 35.5 cm) and foam racks of different heights (2, 4, 6, 8, 10 and 12 cm) were used in this study. A total of twenty four straws were placed in one form rack during freezing. Approximately 4-cm liquid nitrogen was placed in the Styrofoam box and the lid was loosely closed to expel air for 3 min before placing the straws on a rack at the required height from the liquid nitrogen surface. Figure 1 shows the apparats used for liquid nitrogen vapour freezing.

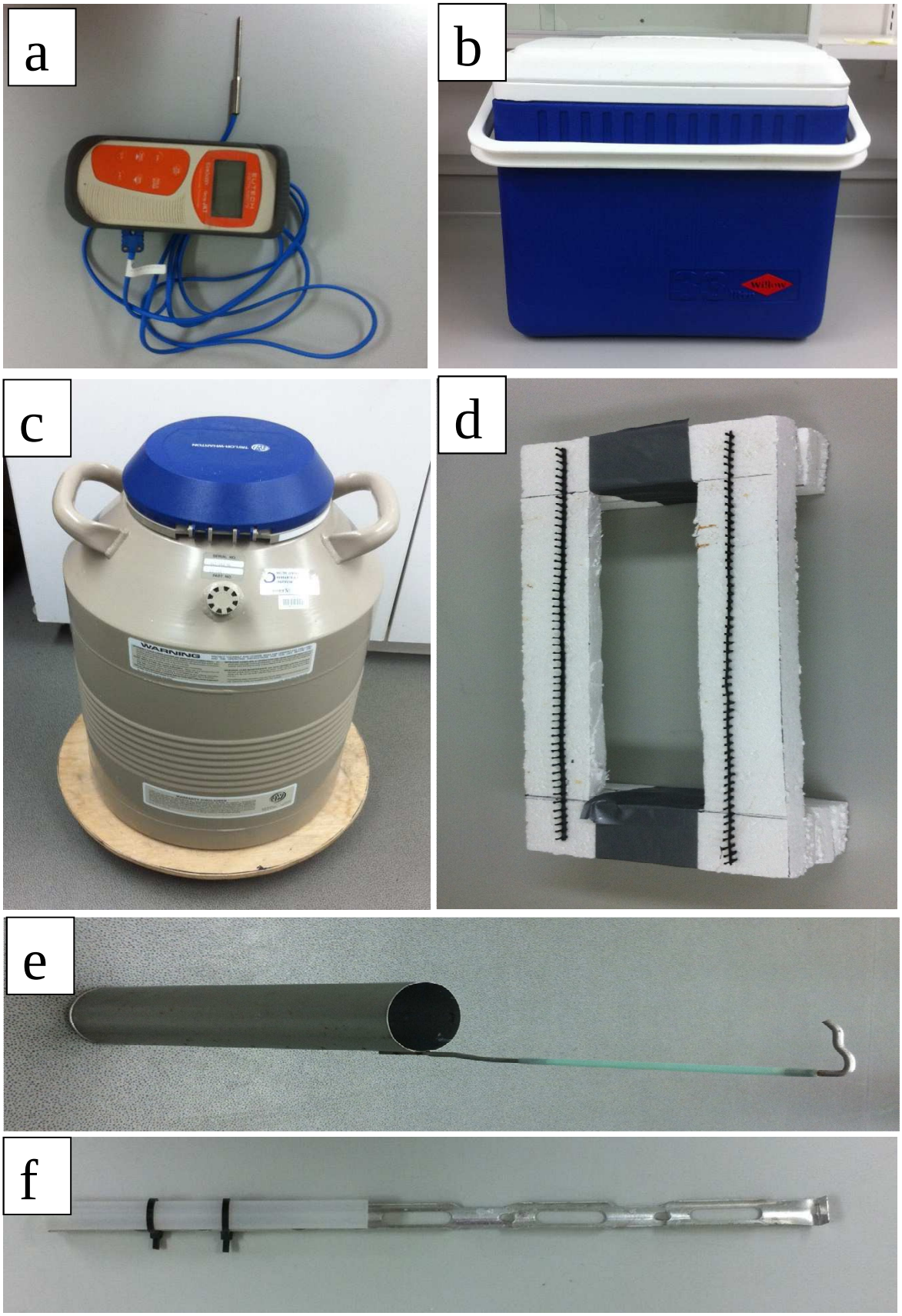
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Fig 1: The apparatus used for liquid nitrogen vapour freezing. (a) digital thermometer, (b) styrofoam box, (c) liquid nitrogen storage dewar, (d) straw holding rack, (e) canister and (f) goblet attached to cryocane.

7.3.3 Sperm collection and quality assessment

The details of sperm collection and quality assessment for this flat oyster species have been described in chapter 6 of this thesis. Briefly, sperm were collected by stripping with a 3.5 ml Pasteur pipette and placed on ice until being used in the study. Collected sperm were filtered through a 45-µm screen, and the sperm concentration was adjusted to 1×10^9 cells/ml after the count with the spectrophotometric method. Sperm motility was blind assessed by two observers and the video was recorded using an Olympus BX50 microscope. The motility percentage from two observers was averaged as mean sperm motility of the subsamples and was validated (only if higher than 10% variation) by video-recorded motility. Sperm from 3-5 males were pooled in a replicate. Post-thaw motility and PMI were used as quality sperm assessment indicators. The forward moving sperm were considered motile but those vibrating in the same place were considered non-motile. The samples with at least 50% fresh sperm motility were used in the subsequent experiments. Dual staining by SYBR 14 and propidium iodide was used to distinguish live and dead sperm. SYBR 14 bound to the DNA of membrane intact sperm emitted green light whereas propidium iodide bound to the DNA of membrane ruptured sperm emitted red light. Therefore, sperm emitted green and red light were considered live and dead, respectively.

7.3.4 Experiments

7.3.4.1 Cryoprotectant toxicity

Four permeable cryoprotectants dimethyl sulfoxide (DMSO), methanol, ethylene glycol (EG) and propylene glycol (PG) were used to evaluate cryoprotectant toxicity. Sperm were equilibrated with each cryoprotectant at 5, 10, 15 and 20% final concentrations for 30, 60 and 90 min, respectively. Sperm motility was used as a toxicity assessment indicator.

7.3.4.2 Cryoprotectant selection

In the first trial, the effect of a permeable cryoprotectant on post-thaw sperm motility was evaluated. Sperm were equilibrated with 5, 10, 15 and 20% of DMSO, methanol, EG and PG, and then cryopreserved. The following cryopreservation protocol was used in this experiment: sperm were equilibrated with each cryoprotectant at a required concentration for 30 min, packaged in 0.25 ml French straws, placed on a foam rack of 8 cm above the liquid nitrogen surface for 20 min, plunged into liquid nitrogen, and then stored in the liquid nitrogen dewar. The cryopreserved sperm were thawed in a 40 °C water bath for 6 sec prior to motility evaluation.

In the second trial, glucose and trehalose were added to DMSO and EG to evaluate the combination effects of permeable and non-permeable cryoprotectants on post-thaw sperm motility. A total of 18 cryoprotectant combinations were evaluated, including 10% DMSO, 10% EG or 15% EG in combination with 0.2, 0.4 or 0.6 M glucose or trehalose. In the third trial, sperm were equilibrated with 15% EG + 0.2 M trehalose for 10, 20, 30 or 60 min before being frozen to evaluate the effect of equilibration duration on post-thaw sperm motility and PMI.

7.3.4.3 The effects of rack height

Sperm were placed on a foam rack at 2, 4, 6, 8, 10 or 12 cm above the surface of liquid nitrogen to evaluate the effect of rack heights on post-thaw sperm motility and PMI.

Temperature at different rack heights were recorded with a digital thermometer (Thermo Scan, Eutech Instruments, Singapore) attached to a low temperature probe. The cooling rates for rack heights 2, 4, 6, 8, 10 and 12 were - 74.3, - 48.7, - 34.6, - 25.8, - 21.3 and - 16.9 °C/min, respectively. Sperm were equilibrated with 15% EG + 0.2 M trehalose for 20 min, packaged in 0.25 ml straws, placed on a rack of the required height above the liquid nitrogen surface for 20 min, and thawed in a 40 °C water bath for 6 sec.

7.3.4.4 The effects of holding duration

Sperm were held on the rack 8 cm above the liquid nitrogen surface for 2, 5, 10 and 20 min to evaluate the effect of holding duration on post-thaw sperm motility and PMI. Sperm were equilibrated with 15% EG + 0.2 M trehalose for 20 min, packaged in 0.25 ml straws, placed on the 8 cm height rack, and thawed in a 40 °C water bath for 6 sec.

7.3.4.5 The effects of sperm volume

The effects of sperm volume on post-thaw motility and PMI were evaluated by freezing sperm in 0.25 ml and 0.5 ml straws. The freezing protocols described in Experiment 7.3.4.4 were used in this experiment. The 0.25 ml and 0.5 ml straws were thawed in a 40 °C water bath for 6 sec and 8 sec, respectively.

7.3.4.6 Comparison of freezing methods

The programmable freezing, vitrification and liquid nitrogen vapor freezing methods were compared based on post-thaw sperm motility and PMI achieved in each method. The protocols for programmable freezing and vitrification methods were obtained from chapter 6 of this thesis. The programmable freezing protocol includes: sperm were equilibrated in 10% DMSO + 0.45 M trehalose for 30 min, packaged in 0.5 ml straws, frozen at -3 ºC/min, and thawed at 40 ºC for 8 sec.

7.3.5 Statistical analysis

To standardize sperm motility in different treatments, fresh motility was set in percent and post-thaw sperm motility was calculated relative to fresh motility. All the percentage data were arcsine transformed prior to statistical analysis. Depending on the number of factors involved, treatment effects on sperm motility and PMI in experiments 7.3.4.1 to 7.3.4.4 were analysed with one-factor or two factors analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT) when differences were significant. A repeated-measure design was applied when data were collected at different time intervals. In experiments 7.3.4.5 and 7.3.4.6, treatment effects on post-thaw sperm motility and PMI were analysed with independent t-test. Data were presented as mean ± SE. The significant level was set at $P < 0.05$. Data were analysed with SPSS version 20.0 (IBM Corporation, Armonk, NY, USA).

7.4 Results

4.4.1 Cryoprotectant toxicity

Motility of sperm exposed to DMSO, EG and PG was similar at all the concentrations and exposure durations evaluated (Fig 2). Sperm motility (%) decreased after 30 min exposure to 10% or higher methanol, and the toxicity of methanol increased with increasing concentration and exposure time.

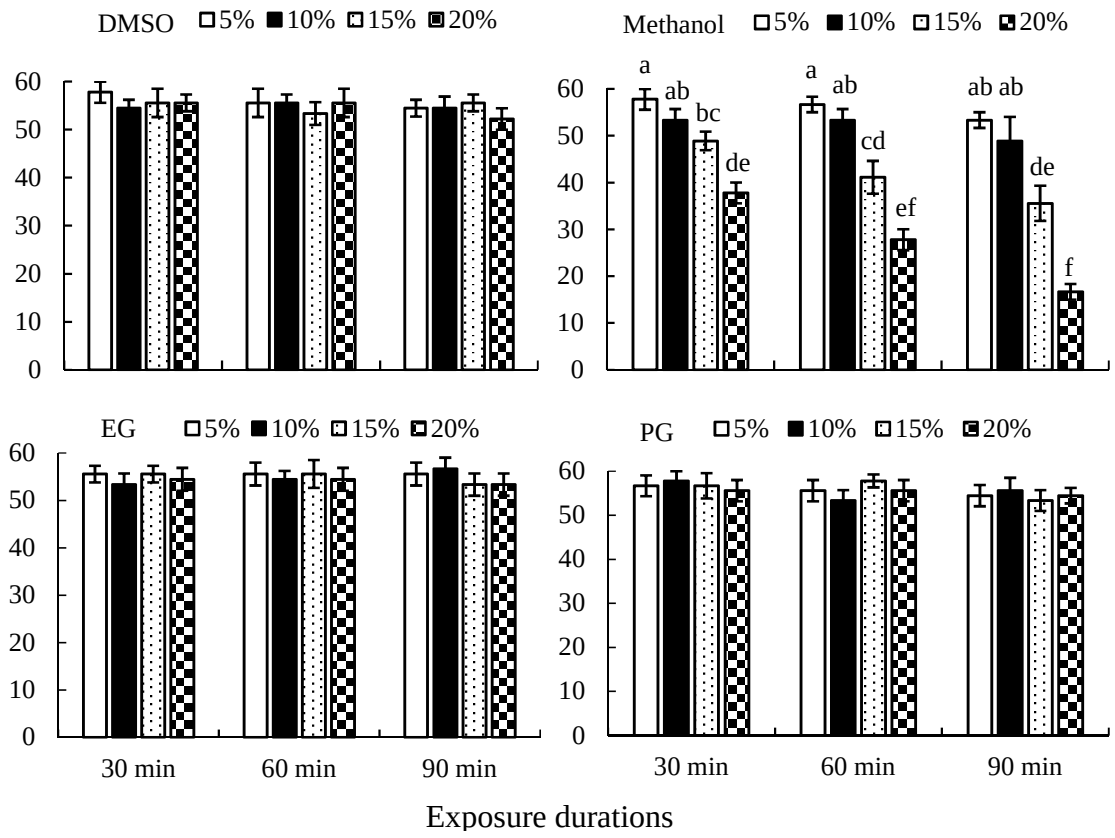

Fig 2: Motility (%) of sperm after exposure to different concentrations of dimethyl sulfoxide (DMSO), methanol, ethylene glycol (EG) and propylene glycol (PG) for 30, 60 or 90 min. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of three replicates. Sperm motility was not significantly different during exposure to DSMO, EG and PG. Different letters in methanol represent significant effect of cryoprotectant concentrations and exposure durations on sperm motility.

7.4.2 Cryoprotectant selection

The post-thaw motility of sperm cryopreserved with different cryoprotectant concentrations were significantly different ($P < 0.05$). The highest post-thaw motility was achieved by cryopreserving sperm with 15% EG, followed by 10% EG and 10%

DMSO (Fig 3). Sperm cryopreserved with other cryoprotectant concentrations had significantly low post-thaw motility ($P < 0.05$).

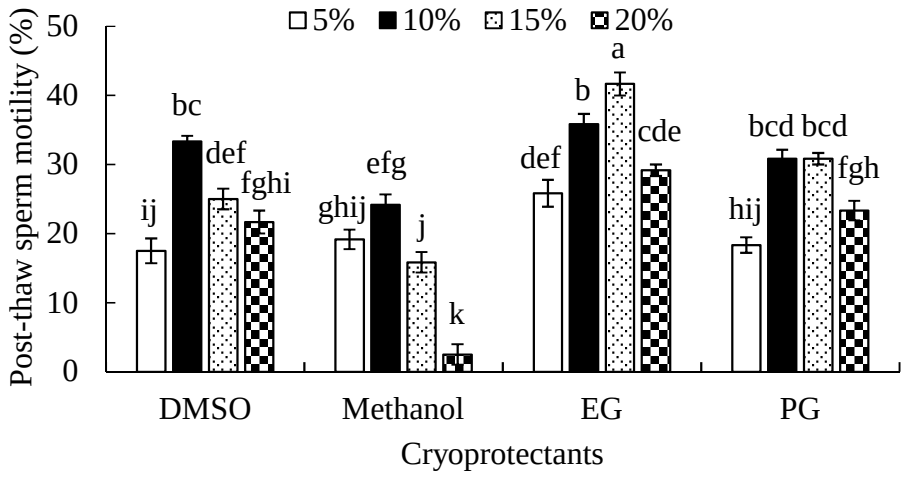

Fig 3: Post-thaw motility (%) of sperm cryopreserved with different cryoprotectant concentrations. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of four replicates. Different letters represent significant effect of cryoprotectants on sperm motility.

The inclusion of glucose and trehalose in DMSO or EG solutions significantly increased the post-thaw sperm motility ($P < 0.05$). The highest motility (%) was achieved by cryopreserving sperm with 15% EG + 0.2 M trehalose, followed by 15% EG + 0.2 M glucose and 15% EG + 0.4 M trehalose (Fig 4).

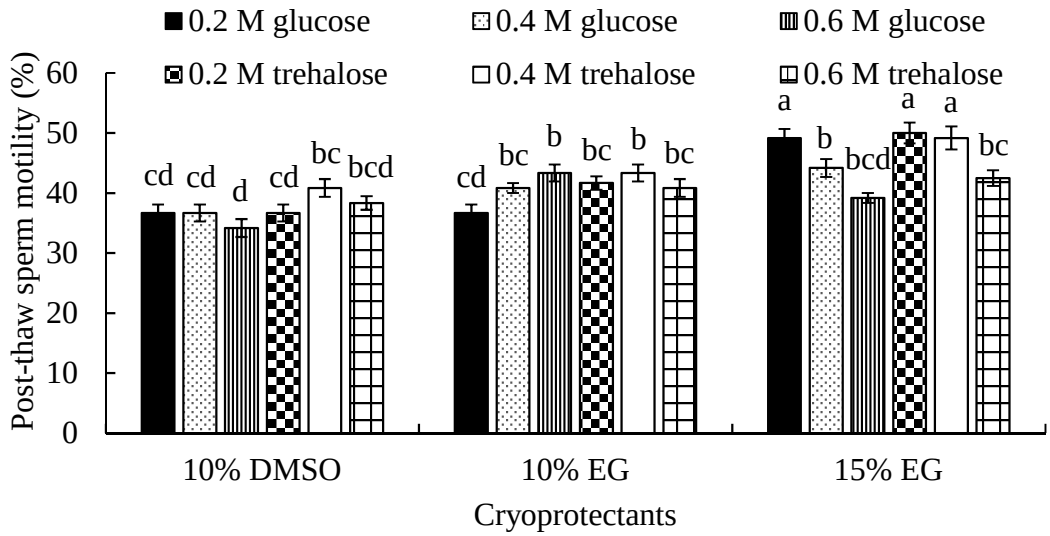

Fig 4: Post-thaw motility (%) of sperm cryopreserved with combinations of permeable and non-permeable cryoprotectants. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of four replicates. Different letters represent significant effect of cryoprotectants on sperm motility. DMSO, dimethyl sulfoxide; EG, ethylene glycol.

The highest post-thaw motility and PMI were achieved in this experiment by equilibrating sperm for 20 min (Fig 5). Sperm cryopreserved at 10 min equilibration produced significantly lower post-thaw motility and PMI than 20, 30 and 60 min ($P < 0.05$). The post-thaw motility and PMI of sperm cryopreserved at 20, 30 and 60 min equilibration were similar.

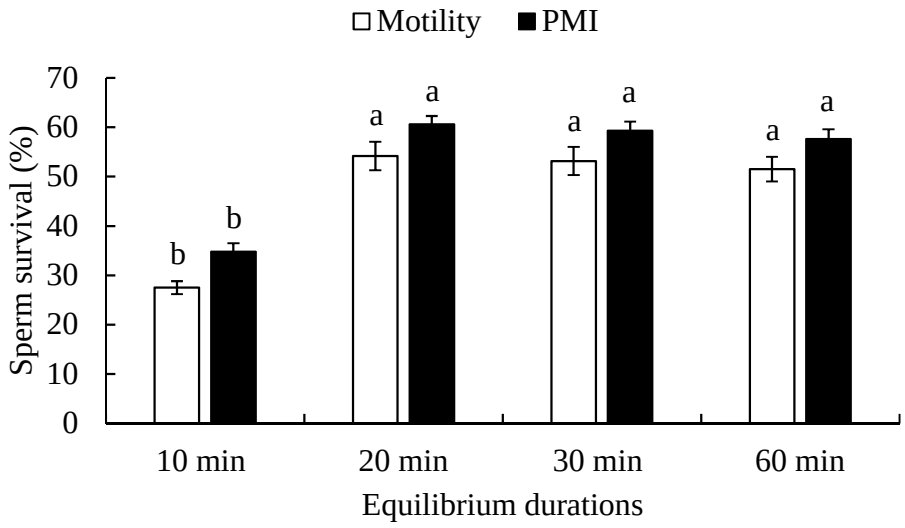

Fig. 5: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved at different equilibration durations. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of four replicates. Different letters represent significant effect of equilibrium durations on sperm motility or PMI.

7.4.3 Effect of rack heights

The highest post-thaw sperm motility (%) and PMI were achieved in this experiment by placing sperm on the 8 cm rack (Fig 6). All the other rack heights used in this experiment produced significantly low post-thaw motility and PMI than the 8 cm rack height ($P < 0.05$).

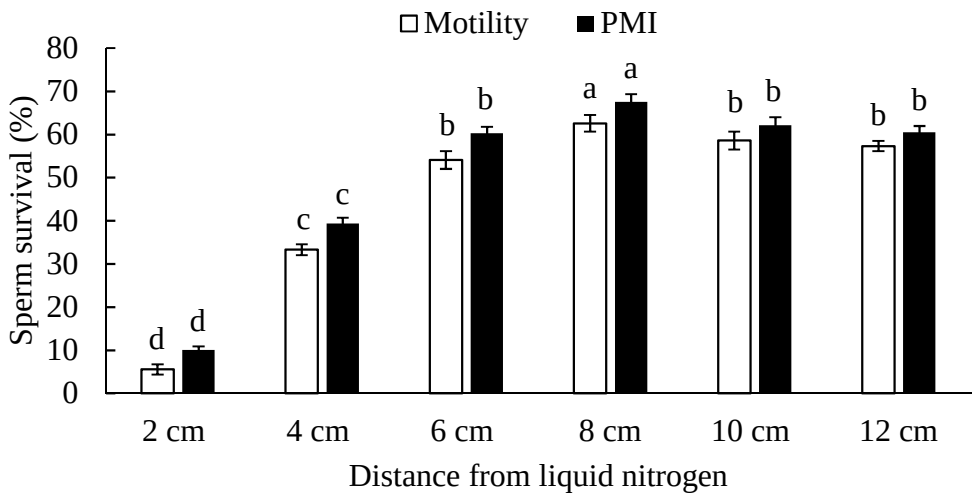

Fig 6: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved at different distances from liquid nitrogen. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of five replicates. Different letters represent significant distance effect on sperm motility or PMI.

7.4.4 Effect of holding durations

The highest post-thaw motility and PMI were achieved in this experiment by holding sperm in liquid nitrogen vapor for 10 min followed by 20 min (Fig 7). The 2 and 5 min holding durations produced significantly lower post-thaw sperm motility and PMI than 10 and 20 min ($P < 0.05$).

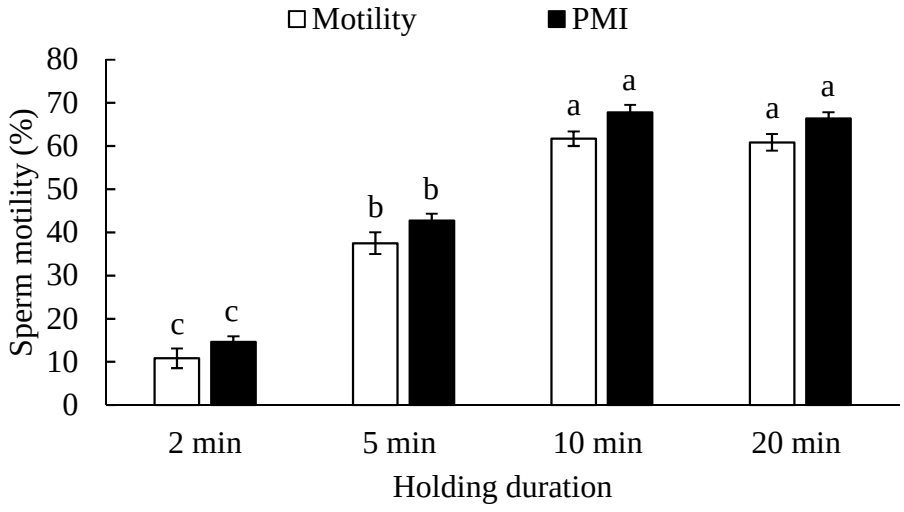

Fig 7: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm frozen at different durations in liquid nitrogen vapour. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of four replicates. Different letters indicate significant effect of holding durations on sperm motility or PMI.

7.4.5 Effect of straw volume

Sperm packed in 0.25 ml straw resulted in better post-thaw motility and PMI than those packed in 0.5 ml straws, although the differences were not significant between them ($P > 0.05$; Fig 8).

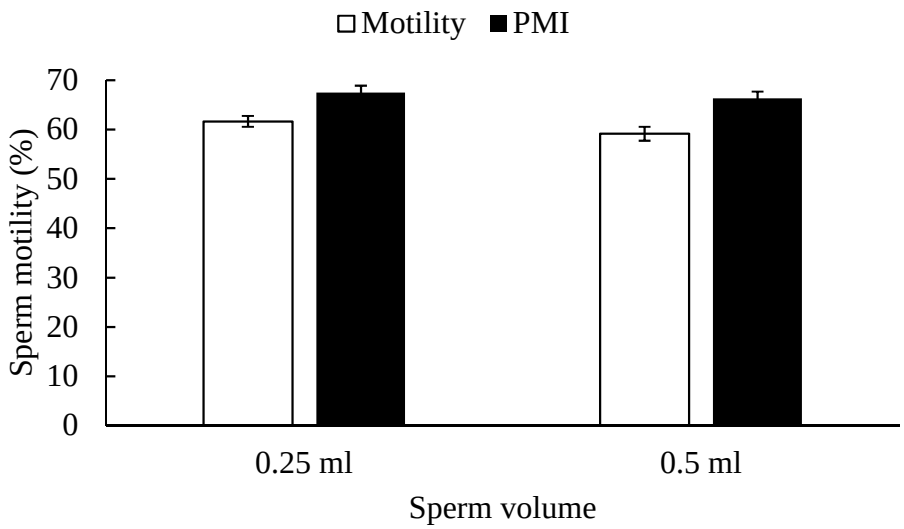

Fig 8: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved in 0.25 ml and 0.5 ml French straws. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of four replicates.

7.4.6 Comparison of freezing methods

The post-thaw sperm motility and PMI were significantly affected by the freezing methods evaluated ($P < 0.05$). The post-thaw sperm motility was 44.4% and 62.2%, and

PMI was 49.2% and 67.3% in programmable freezing and liquid nitrogen vapor freezing methods, respectively (Fig 9).

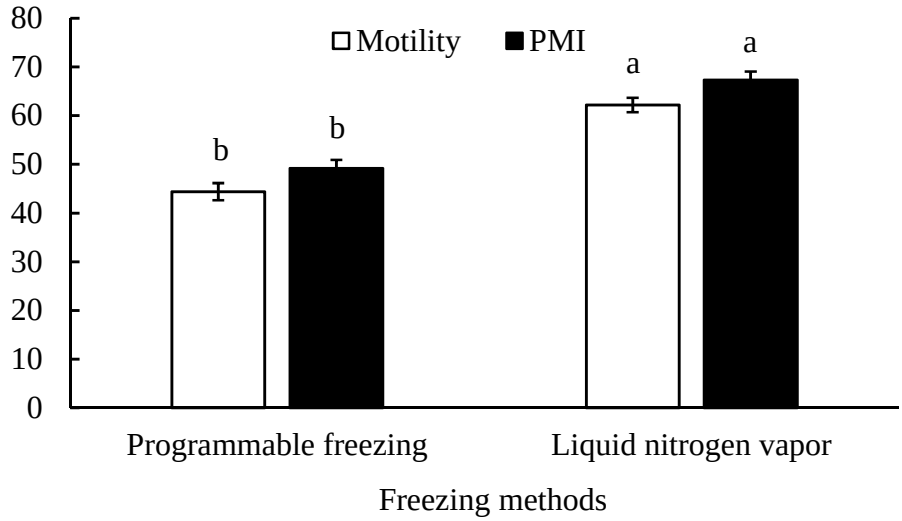

Fig 9: Post-thaw motility (%) and plasma membrane integrity (PMI) of sperm cryopreserved with programmable freezing and liquid nitrogen vapor methods. Sperm from three to five males were pooled in a replicate. Each bar represents mean ± SE of three replicates. Different letters indicate significant effect of freezing methods on sperm motility or PMI.

7.5 Discussion

This study optimized the key factors for cryopreservation of the Australian flat oyster sperm using the method of liquid nitrogen vapor freezing. The liquid nitrogen vapor method increased both post-thaw motility and PMI by 15% over the programmable freezing method, and by 10-fold over the vitrification method. The ability of cryopreserved sperm to fertilize egg is the ultimate criterion for sperm quality assessment but fertilization assessment was not applicable to this species due to lack of a reliable method for artificial egg fertilization. Post-thaw motility and PMI were used as indicators to assess sperm quality in this study because these two variables have

strong correlations with fertility in published references (Au et al., 2002; Moskovtsev et al., 2005).

Cryoprotectants might be toxic to sperm with the increase in their concentrations and exposure durations, therefore the selection of a cryoprotectant normally initiates with toxicity evaluation. The suitability of a cryoprotectant is also species specific and a cryoprotectant suitable for one species might be toxic to another in a taxonomic group. Surprisingly, up to 20% of DMSO, EG and PG were non-toxic to the sperm of Australian flat oysters after 90 min equilibration. Although DMSO, EG and PG are suitable for sperm cryopreservation in many marine invertebrates, they are generally toxic at the concentration of 15% or higher and the exposure duration of 30 min or longer (Gwo, 2000; Liu et al., 2015a). Sperm of this flat oysters have exceeded the toxicity tolerance limits of other marine invertebrate species reported so far for these three cryoprotectants. However, 10% methanol or higher was toxic to this species, which is similar to the toxicity reported in the European flat oyster (Vitiello et al., 2011), mangrove oyster (Nascimento et al., 2005) and Mediterranean mussel (Di Matteo et al., 2009).

The permeable cryoprotectants could enter the cell and improve its osmotic balance, thereby reduce the chance of intracellular ice crystal formation, and minimize freezing injury to the cell. A lower concentration and shorter exposure duration of a cryoprotectant might be insufficient to bring an equilibrium between extracellular and intracellular solutes, but a higher concentration and longer exposure duration might cause a solute effect (Meryman et al., 1977). Both these consequences are deleterious to sperm survival, therefore delicate adjustment between the osmotic balance and solute effect would provide better protection from freezing injury. In this study, 15% EG, 10% EG and 10% DMSO were suitable cryoprotectant concentrations for the Australian flat oyster sperm. The other cryoprotectant concentrations evaluated in this study were

either insufficient to protect sperm or imposed solute and toxic effects. EG was also a suitable cryoprotectant in other marine invertebrates, but the optimum concentration might differ between species, i.e., 15% EG in the European flat oyster (Vitiello et al., 2011) and 7% EG in Mediterranean mussel (Di Matteo et al., 2009). DMSO is the most widely used cryoprotectant in aquatic species, with the optimal concentration range of 5-20% being found in different species (Martínez-Páramo et al., 2016).

Addition of non-permeable cryoprotectants to permeable cryoprotectants could provide better osmotic balance and cell membrane cohesiveness, and minimizes ice crystallization. This strategy has been widely used to improve cryopreserved sperm quality in different animal clades including mammals (Aboagla & Terada, 2003; Gómez-Fernández et al., 2012), teleosts (Ciereszko et al., 2014; Nynca et al., 2016) and invertebrates (Acosta-Salmón et al., 2007; Liu et al., 2014a). In this study, addition of glucose or trehalose to DMSO and EG improved the post-thaw sperm motility, and the combination of 15% EG + 0.2 M trehalose produced the highest sperm motility. In other marine invertebrates, the highest post-thaw motility was achieved with the combination of 8% DMSO + 0.25 M trehalose and 5% DMSO + 0.5 M trehalose in the Chilean oyster (Adams et al., 2013), 5-12% DSMO + 0.45 M trehalose in the Pacific oyster (Adams et al., 2004), 5% DMSO + 1 M trehalose in the black-lip pearl oyster (Lyons et al., 2005). These results suggest that the types and concentrations of permeable and non-permeable cryoprotectants are species specific.

The freezing is a sensetive step in the cryopreservation process. While a faster freezing induces intracelluar ice formation due to insufficient time for excess water to leave the sperm, a slower freezing causes solute effect due to exposure to the cryoprotectant medium for a longer period. Thereby, a balance is needed for the cell viability. In this study, the height of 8 cm from the liquid nitrogen surface resulted in the highest post-thaw motility and PMI of all heights evaluated, which differs from the

optimal heights reported for other marine invertebrates. For example, the hight of 3 cm was found to be the optimal in the black-lip pearl oyster (Hui et al., 2011), 5.2 cm in the green-lip abalone (Liu et al., 2014b) and black-lip abalone (Liu et al., 2015b), and 9-12.5 cm in the Japanese pearl oyster (Kawamoto et al., 2007; Arita et al., 2012). Athough the optimal height differs among speceis, the results from this and published studies suggest that the optimal height range is narrow in the species investigated so far.

The holding duration on a rack (the period in which samples are placed in liquid nitrogen vapor before being plunged in liquid nitrogen) affect the viability of cryopreserved sperm. The holding duration changes with the endpoint temperature and allows cryoprotectants to equilibrate between inter- and extra-celluar media. The highest post-thaw motility and PMI were achived by holding sperm for 10 min in liquid nitrogen vapor although it was not significantly different from those held for 20 min, suggesting that the holding duration from 10 to 20 min would be suitable for sperm cryopreservation in this species. The period of 10-min holding was also applcable for cryopreservation of greeen-lip abalone, black-lip abalone and black-lip pearl oyster (Hui et al., 2011; Liu et al., 2014b; Liu et al., 2015b).

Straw volume is important from both cryobiological and applicational aspects. Regardless of the freezing and thawing procedures, the straw volume determines the actual heat transfer rate. A high-throughput automation for commercial scale application can be achieved with 0.25 ml and 0.5 ml French straws (Yang et al., 2007; Yang et al., 2012) but a larger volume (e.g., 4.5 ml cryovials) has greater application in small and medium scale hatchery operations where such automation is unavailable (Adams et al., 2008). Interestingly, the effect of straw size in cryopreservation differs among studies, probably due to the interaction of other factors such as freezing and thawing rates. In this study, there was no significant difference in post-thaw sperm motility and PMI between sperm samples cryopreserved in 0.25 ml and 0.5 ml straws. However,

significantly higher post-thaw motility and PMI were achieved in 0.5 ml straws compared with those in 0.25 ml straws in this species using a programmable freezing method described in chapter 6 of this thesis. Straw volume has no significant effect on post-thaw sperm motility in the Pacific oyster as well (Dong et al., 2005). In contrast, significant differences in post-thaw sperm motility were found between straw volumes in boar, rainbow trout and Japanese pearl oyster (Cabrita et al., 2001; Aoki et al., 2007; Buranaamnuay et al., 2009).

Although freezing methods could affect sperm viability (Varadi et al., 2013), this aspect has rarely been investigated in marine invertebrates. Sperm cryopreservation protocols have been developed from independent studies using a range of freezing methods including laboratory-scale programmable freezing (Di Matteo et al., 2009), commercial-scale programmable freezing (Dong et al., 2007; Yang et al., 2012), liquid nitrogen vapor freezing (Liu et al., 2015b), and methanol - dry ice (Adams et al., 2004). In comparions between freezing methods, liquid nitrogen vapor produced significantly higher post-thaw motility and PMI than those cryopreserved with programmable freezing. Although both programmable freezing and liquid nitorgen vapour freezing have produced high post-thaw motility and fertility in marine molluscs (reviewed by Liu et al., 2015a), the results of this study indicate a higher sensitivity of sperm to programmable freezing.

In conclusion, 62.2% post-thaw motility and 67.3% PMI were achieved in this study using the liquid nitrogen vapor method. The protocol requires that sperm are equilibrated in 15% EG + 0.2 M trehalose for 20 min, packaged in 0.25 ml straws, placed on a foam rack of 8 cm above the liquid nitrogen surface for 10 min, and then immediately plunged into liquid nitrogen. By comparing the post-thaw motility and PMI achieved in programmable freezing technique, the study concludes that the liquid

nitrogen vapor freezing technique is an effective method for the cryopreservation of Australian flat oyster sperm.

7.6 Acknowledgments

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Chapter 8: General Discussion and Conclusion

8.1 Introduction

Harvest of wild fishery in the ocean has shown a declining trend in the last two decades, but marine aquaculture is expanding to increase global seafood supply. While intensification of aquaculture systems is filling the gap in seafood production, anthropogenic inputs and climate changes are imposing pressure on the health and sustainability of marine ecosystems. The activity of filter-feeding in marine bivalve species can reduce sediment load and algal bloom, convert primary production to benthic secondary production, and increase system productivity, therefore aquaculture of marine bivalve species is an alternative option to sustain marine resources (Welsh, 2003). Long term sustainability in aquaculture of a marine bivalve species depends on growth rates and disease resistance. The development of breeding programs to increase growth rate and disease resistance would increase competitiveness of a bivalve species in aquaculture.

Spermcasting oysters are important aquaculture candidates globally, but very limited progress has achieved in their breeding programs to develop traits of aquaculture need (Newkirk, 1986). One of the main obstacles for the development of breeding programs in spermcasting oysters is their unique reproductive strategy of incubating fertilized eggs and larvae inside the body cavity. Comprehensive knowledge on reproductive strategy is a fundamental requirement for designing a breeding program of a species. Availability of complementary techniques such as sperm quantification and cryopreservation also contributes to breeding programs by providing opportunities for controlled and/or off-season breeding. In addition, preservation of gametes from superior broodstock and stocks with desired genetic makeups is important to protect the breeding populations from epidemic disease and natural disaster, and to provide a reference for evaluating cross-generation genetic progress. Considering these aspects, this thesis aims to increase the capacity and efficiency in breeding programs of

spermcasting species by contributing new knowledge to spermcast spawning and by developing techniques for sperm quantification and cryopreservation of a spermcasting bivalve species, the Australian flat oyster *Ostrea angasi*. This discussion chapter integrates the major findings of this thesis, identifies the knowledge advances, and provides future research directions based on the results of this study.

8.2 Summary of major findings

1. *The levels of masculinity in a flat oyster determine the functionality of male gametes*. Spermatogonia gradually aggregated in the gonad follicles at the early stages and became denser and more compacted at the advanced stages. In hermaphroditic flat oysters, the proportion of gonadal volume occupied by spermatozeugmata ranged from only a few clusters to almost the entire gonad. The functional properties of spermatozeugmata differ among individuals according to their masculinity levels. Spermatozeugmata of a true male have greater capacity to maintain spermatozeugmata integrity and sperm motility than a hermaphrodite, and spermatozeugmata of a male-dominated hermaphrodite have greater capacity to maintain spermatozeugmata integrity and sperm motility than a female-dominated hermaphrodite.

2. *The spermcasting oyster has unique reproductive characteristics in gametogenesis, sex ratio and energy metabolism.* The population of 2-3 years old *O. angasi* has a high percentage of hermaphrodites (46.7%) and males (41.3%). During gametogenesis both male and female gametes matured at a similar time in a hermaphroditic individual but the gonad texture of partially spawned individuals indicated that spermatozeugmata were released before egg ovulation. Glycogen was the main energy reserve for gametogenesis, and *O.*

angasi followed an intermediate strategy in energy metabolism between conservative and opportunistic species.

3. *The method of spectrophotometry can reliably estimate sperm density in spermcasting oysters*. The peak of absorbance for *O. angasi* sperm occurred at 350 nm wavelength in spectrophotometry, and the best predicting model is expressed as $y = 1\times10^{-8} x + 0.163$, where x and y denote sperm concentration (cells/ml) and absorbance, respectively. The model prediction for sperm counts agrees with the result from the haemacytometry method.

4. *Development of a new protocol using liquid nitrogen vapour to improve sperm survival*. The inclusion of non-permeable cryoprotectants with permeable cryoprotectants improved sperm survival in both programmable computer-controlled freezing and non-programmable liquid nitrogen vapour freezing methods. However, the method of liquid nitrogen vapour freezing produced significantly higher survival of post-thaw sperm than a computer-controlled freezing. The new protocol for cryopreservation using liquid nitrogen vapour required that sperm equilibration in 15% ethylene glycol + 0.2 M trehalose for 20 min, package in 0.25 ml straws, placement on a foam rack of 8 cm above the liquid nitrogen surface for 10 min, and plunge immediately into liquid nitrogen.

8.3 Knowledge advance and study significance

8.3.1 Discovery of spermatozeugmata structure and functional properties

Fertilization success of sessile species depends on the transfer of male gametes to the body cavity of a female, and factors such as water flow, population density, and sperm motility affect fertilization success (André & Lindegarth, 1995; Bishop, 1998; Hodgson et al., 2007). This study identifies the structure and functional properties of male

gametes and their implications for successful fertilization of spermcasting species. The male gametes in spermcasting species are clustered in a capsule known as spermatozeugma which is bounded by a gelatinous membrane. In previous studies, spermatozeugmata dimension and swimming pattern have been described (Foighil, 1989; Falese et al., 2011). But this study further advances the existing knowledge of spermatozeugmata biology by providing key information on the development of spermatozeugmata in the early stage, sperm structure, pattern of spermatozeugmata dissociation and gamete longevity. Interestingly, this study discovered that the spermcasting *O. angasi* sperm has five mitochondria whereas other broadcasting species have four mitochondria. Mitochondria supply energy for sperm movement, and an additional mitochondrion may be used to supply more energy for spermatozeugmata movement. The additional mitochondrion may be evolutionarily important towards reproductive adaptation to support sperm swimming in spermcasting species.

This study extended our understanding on the correlation between functional properties of spermatozeugmata and the level of masculinity in the spermcasting oyster. A true male can maintain longer spermatozeugmata integrity and sperm motility than a hermaphroditic individual, and the male-dominated hermaphrodite can maintain longer spermatozeugmata integrity and sperm motility than the female-dominated hermaphrodite. This finding has important implications in fertilization success of spermcasting species. When brooders are far apart, spermatozeugmata produced by hermaphrodites would have a lower chance to fertilize eggs than those produced by pure males.

8.3.2 Gametogenesis, sex ratio and energy metabolism

The patterns of gametogenesis, sex ratio and energy metabolism have important implications in reproductive and physiological adaptations in marine bivalves. The proportion of gametes during gametogenesis in hermaphroditic *O. angasi* varied from a highly-skewed in one sex to equal proportions in both male and female gametes. This study identifies the developmental asynchrony of male gametes and developmental synchrony of female gametes during gametogenesis. The partially-spawned hermaphrodite individuals also indicated the release of male gametes prior to female gamete ovulation. The differences in the time window between spawning events of male and female gametes is crucial to avoid self-fertilization in hermaphrodites. Spermatozeugmata acquisition prior to egg ovulation also ensures fertilization success. Otherwise, the embryonic development may fail if the maternal adult ovulates eggs before spermatozeugmata acquisition (Stewart-Savage et al. 2001).

Although oysters in the family Ostreidae are protandric hermaphrodites, the sex ratio varies among the broadcasting and spermcasting species. A very high proportion of hermaphrodites (46.7%) in 2-3 years old *O. angasi* in this study confirms the pattern of protandric reproduction in spermcasting oysters. The high percentage of hermaphrodites in a spawning population could have adaptive advantage to maintain population by using either the male or female function during fertilization.

Majority of marine bivalves follow a conservative strategy of energy metabolism by keeping energy in storage tissues prior to gametogenesis (Darriba et al., 2005; Li et al., 2006; Karray et al., 2015), but few others follow an opportunistic strategy by providing energy for gametogenesis directly from consumed food (Ruiz et al., 1992). This study provides new findings of how a marine bivalve species metabolizes energy to support a long spawning period with low caloric intake. In *O.*

angasi living in a low productive habitat with a long spawning period, glycogen serves as the main energy reserve for gametogenesis though protein can be an additional source of energy reserve especially during the period of low glycogen reserve. Interestingly, this species displayed an intermediate strategy in energy metabolism between conservative and opportunistic species. Because gametogenesis is a high energy demanding process, neither the conservative nor opportunistic strategy may be sufficient to meet the energetic demand for long spawning periods in a low food habitat. Therefore, the intermediate energy metabolism strategy is used as an adaptation to meet the energetic demand of gametogenesis from both consumed food and energy reserve in the body.

8.3.3 Development of a spectrophotometric technique for sperm quantification

Spectrophotometry can be applied to develop a relatively simple, cheap and fast technique for sperm quantification, and has been applied to fish and broadcasting marine invertebrates. This study further extends the application of spectrophotometry by developing a regression model for sperm quantification in spermcasting species. Due to species specificity, the most suitable wavelength for generating a regression model was found at the 400-nm wavelength in the Atlantic croaker and small bodied fishes (Tan et al. 2010; Leclercq et al. 2014), 320 nm in the blue mussel (Miguel and Beaumont, 2008), and 581 nm in the Pacific oyster (Dong et al. 2005). This study identified the suitable wavelength of 350 nm for *O. angasi*, and developed a regression model accordingly. Absorbance of 30 samples can be obtained within 10 sec on a 96-well plate to calibrate sperm concentrations using the regression model $y = 1\times10^{-8} x + 0.163$, whereas 10-15 min is required to count sperm per sample using a haemacytometer. The

spectrophotometric technique for sperm quantification could increase the efficiency for a breeding program and cryopreservation in spermcasting species.

8.3.4 Development of new cryopreservation protocol

Sperm cryopreservation in oysters has developed for nearly 50 years since the first successful practice on Pacific oyster sperm (Lannan, 1971), but past research was mainly focused on broadcasting species (Hassan et al., 2015). The sensitivity of sperm in cryopreservation of broadcasting and spermcasting species are different due to variations in spermatological property. This study advances the knowledge on cryopreservation of spermcasting species by systematically optimizing different steps using various freezing approaches. By comparing sperm survival, this study provides further evidence that liquid nitrogen vapour is the most effective option for cryopreservation of *O. angasi* sperm. In addition, this study has advanced the understanding that the inclusion of non-permeable cryoprotectants with permeable cryoprotectant improves sperm survival irrespective of freezing methods. In previous studies on spermcasting oysters, the survival rate of cryopreserved sperm was relatively low (Vitiello et al., 2011; Horváth et al., 2012). This study improved sperm survival by optimizing cryoprotectants, sample distance from the liquid nitrogen surface, holding duration of sperm in liquid nitrogen and sample volume. The new understanding of cryopreservation provides advanced skills in cryobiology research, and the new protocol would increase the efficiency for future breeding and genetic improvement programs in spermcasting species.

8.4 Conclusions

This thesis studied the reproductive strategies of a spermcasting species and developed techniques for sperm quantification and cryopreservation. The outcomes of this research

contribute to a better understanding on the life history of spermcasting species and improvement of efficiency in breeding programs for sustainable aquaculture development of spermcasting bivalves. The following major conclusions are drawn based on the research findings of my study:

1) Sperm heads are clustered in a spermatozeugma, but sperm can become a free-swimming individual after dissociation of the spermatozeugma membrane. The structural and functional properties of spermatozeugmata have significant adaptations to enhance fertilization success in spermcasting species.
2) The difference in time window for releasing male and female gametes in hermaphroditic species could be a significant adaptation to avoid self-fertilization. The time difference of gamete release could ensure acquisition of functional spermatozeugmata by a maternal adult for egg fertilization.
3) The intermediate strategy of energy metabolism by acquiring energy from reserved energy in body tissues and external energy from food consumption during gametogenesis has adaptive significance for *O. angasi* that experience a long period of gamete production in a habitat with low food supply.
4) Spectrophotometry is a rapid and reliable technique for sperm quantification and can increase the efficiency of cryopreservation in a breeding program. The regression model based on *O. angasi* for sperm quantification should be validated before applying to a new species.
5) Sperm of spermcasting oysters are more sensitive to cryopreservation than broadcasting oysters, but sperm injuries can be reduced by mixing non-permeable and permeable cryoprotectants. The liquid nitrogen vapour is a better option than the programmable-controlled freezing method in cryopreservation of *O. angasi* sperm.

8.5 Implication of the research results in aquaculture and breeding programs

Similar to other aquaculture species, long-term success for aquaculture of spermcasting species would largely depend on the development of breeding programs. The knowledge and techniques established in this thesis would assist the industry development, especially in breeding and genetic improvement programs in the following areas:

1) Spermatozeugmata of paternal adults with different masculinity levels vary in reproductive functionality. A non-invasive gonad sampling technique is useful to identify the masculinity level of a brood. In a controlled-breeding operation, a higher fertilization rate can be achieved by using spermatozeugmata of pure males than those of hermaphrodites.
2) Spermatozeugmata gradually lose motility and functionality in seawater, therefore fertilization efficiency of spermatozeugmata will reduce over time. Minimizing the physical distance between brooders in a breeding tank would increase the chance of spermatozeugmata acquisition by a maternal adult.
3) The Australian flat oyster has natural spawning period from May to December in South Australia, therefore breeding programs can be implemented over an extended period to meet the demand of spat supply by growers.
4) The spectrophotometric technique could be used for sperm quantification in complex breeding programs to increase the efficiency of breeding programs.
5) Genetic uniformity of inbred lines is an ideal approach for genetic mapping. As spermcasting oysters can change sex over time, self-fertilization can be achieved by using cryopreserved sperm to fertilise eggs of the same individual.

6) Cryopreservation provides a continuous gamete supply from the same individual. This would allow backward selection and comparison of genetic gain across multiple generations.

8.6 Future research directions

This research has explored some important aspects of spawning strategies in spermcasting species, and developed techniques for sperm quantification and cryopreservation. However, some questions remain outstanding as they are beyond the scope of this study and need further research, including:

1) As the technique for artificial fertilization is not available in spermcasting species, I was unable to directly compare egg fertilization success using spermatozeugmata obtained directly from the gonad and from cryopreservation during the course of this PhD study. Future research should be directed to compare the fertilization efficiency between fresh sperm and those from cryopreservation in spermcasting bivalves.
2) Manipulative experiments in temperatures, food supply, age group and brooder density would add further understanding of reproductive success in spermcasting oysters.
3) Since spermcasting species has a varying degree of hermaphrodites from a highly-skewed single sex to equal proportions in both sexes, there is a need to test the role of sex transition in hermaphroditic individuals to further improve our understanding on their reproductive biology.
4) Spermcasting oysters incubate fertilized eggs and larvae inside the maternal body cavity, and this is one of the main obstacles to make a breakthrough in breeding programs. A new technique is highly needed to incubate fertilized eggs or early larval stages in the ambient environment. Recent research has suggested

the conditions required for larval incubation inside the shell cavity (Mardones-Toledo et al., 2015; Andrade-Villagrán et al., 2017), and these findings could provide a starting point to further explore the possibility of incubation of eggs and larvae outside the female oyster.

5) The survival rate of cryopreserved sperm in spermcasting oysters is lower than broadcasting oysters and other aquatic species. The reasons of low sperm survival are not yet known. Future research is warranted to unveil the causes of extra sensitivity and to improve survival of cryopreserved sperm. Supplementations of sugars, amino acids and vitamins with cryoprotectants have improved sperm survival in livestock (Memon et al., 2013), fish (Cabrita et al., 2011) and other bivalves (Liu et al., 2014), therefore future research should consider integrating these approaches in spermcasting oyster species.
6) The use of computer assisted sperm analysis to evaluate additional variables such as sperm movement velocity and ATP activity in sperm cryopreservation should be applied in future research on spermcast bivalve species.

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