Sperm Cryopreservation in Australian Farmed Greenlip (*Haliotis laevigata*) and Blacklip (*H. rubra*) Abalone

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DECLARATION

I certify that this work contains no material which has been accepted for any other degree or diploma in any university or institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Yibing Liu

October

2014

ABSTRACT

Greenlip *Haliotis laevigata* and blacklip *H. rubra* abalone as well as their hybrid are the major abalone farmed in Australia. To ensure the long-term sustainable and competitive development of the industry, genetic improvement programs have been applied. However, the efficiency of these programs has been compromised due to: (1) asynchronous spawning in males and females within and between species; (2) the short spawning window period in a breeding season; and (3) risks associated with keeping superior broodstock alive and healthy. Sperm cryopreservation is a proven technique to overcome similar issues and sperm collected from wild greenlip abalone has been investigated and 90% post-thaw sperm fertilization rates have been achieved. Nevertheless, when the developed protocol was applied to sperm from farmed stocks, low and highly variable results were observed, hindering its application. The Australian abalone aquaculture industry now relies entirely on domesticated farmed abalone as broodtsock. Therefore, understanding factors causing this discrepancy, and development of sperm cryopreservation techniques suitable for Australian farmed abalone species, are the focus of this PhD project.

Factors affecting sperm cryopreservation and strategies to improve the sperm quality were evaluated using both programmable and/or non-programmable freezing techniques in farmed greenlip and blacklip abalone. In greenlip abalone, broodstock physiological conditions, such as broodstock age and sperm collection time over a natural spawning period, were also evaluated.

Among the single cryoprotectant agent [dimethyl sulfoxide (DMSO), propylene glycol, ethylene glycol, and glycerol] evaluated, DMSO produced the best post-thaw sperm motility and/or fertilization rates using both programmable and non-

programmable freezing techniques in farmed greenlip abalone. However, to achieve the highest post-thaw sperm fertilization rates of 60 to 70%, a higher DMSO concentration and a sperm to egg ratio were required in the former technique (10% and 40,000:1) than in the latter one (6% and 10,000:1). The addition of sugar (glucose, sucrose or trehalose) in DMSO to further improve the post-thaw sperm quality showed that the post-thaw sperm motility was significantly improved by the addition of 4% sucrose using programmable freezing technique, whereas the post-thaw sperm fertilization rates were not. In contrast, when a non-programmable freezing technique was used, the addition of 1% glucose in 6% DMSO significantly improved both post-thaw sperm motility and fertilization rates, with the latter reaching 80%. Fluorescent stain analyses revealed that the addition of glucose significantly improved the post-thaw sperm plasma membrane integrity (PMI) and mitochondrial membrane potential (MMP).

Data from the addition of amino acids (taurine and glycine) and vitamin (L-ascorbic acid) revealed that the addition of 0.6% glycine in 10% DMSO or 6% DMSO + 1% glucose significantly improved the post-thaw sperm fertilization rates to over 90% when using the programmable and non-programmable freezing techniques, respectively. The addition of glycine improved the post-thaw sperm MMP with application of the former technique, whereas PMI and acrosome integrity (AI) with application of the latter one. After the addition of 0.6% glycine, the optimal sperm to egg ratio remained the same in programmable freezing technique whereas this ratio reduced from 10,000:1 to 2,000:1 in the non-programmable freezing technique. Flow cytometry analyses showed higher post-thaw sperm PMI, MMP and AI values were achieved when using non-programmable verses programmable freezing techniques.

Evaluation of the effects of glucose, fructose and galactose using the non-

programmable freezing technique indicated that in farmed greenlip abalone, galactose and fructose had a similar ability to protect sperm from cryoinjury as glucose. The replacement of glucose with either of these monosaccharides resulted in a similar level of post-thaw sperm fertilization rate, PMI, MMP and AI.

Results from evaluation of broodstock physiological conditions in farmed greenlip abalone showed that sperm collected at the middle of a natural spawning period had a better ability to tolerate the cryopreservation processes than those collected at the beginning or at the end of a natural spawning period, resulting in significantly higher post-thaw sperm motility, fertilization rate, PMI, MMP and AI. In contrast, little difference in these parameters was found between 2 and 3 years old animals.

Among the single cryoprotectant agents assessed in farmed blacklip abalone using the non-programmable freezing technique, 6% DMSO achieved the highest post-thaw sperm motility, which was the same as found for farmed greenlip abalone. Further addition of sugar (glucose, sucrose or trehalose) in 6% DMSO showed that the addition of 2% glucose significantly improved the post-thaw sperm quality, producing the highest post-thaw sperm fertilization rate of 70% at a sperm to egg ratio of 10,000:1, although the rate was significant lower than the control (83%).

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CHAPTER 1 : GENERAL INTRODUCTION

Abalone is a highly valuable species in the international market and before the year 2000, most abalone were harvested from the wild fisheries (Allsopp et al., 2012; FAO, 2012). However, due to over fishing and a large recreational catch, the wild production has declined. The production from aquaculture, on the contrary, has increased since 2000 and its percentage relative to the wild sector has continuously increased over the past decade, contributing greatly to the world abalone production (Flores-Aguilar et al., 2007; FAO, 2012).

Abalone aquaculture started in the United States and Japan due to the relative scarcity and high price (Mcbride, 1998; Flores-Aguilar et al., 2007). The abalone aquaculture industry has now been well established worldwide and several species are farmed in many countries and regions such as China, Chinese Taiwan, Korea, Australia, Chile, Mexico, New Zealand, South Africa, Thailand and the United States (Tables 1.1 and 1.2; Freeman, 2001; Nie and Wang, 2004; Flores-Aguilar et al., 2007; Searcy-Bernal et al., 2010; Allsopp et al., 2012; FAO, 2012). Culture techniques, including hatchery, nursery and grow-out, coupled with environmental and nutritional requirements have been developed and optimized to meet the specific conditions where abalone are farmed (Spencer, 2002). In order to ensure the long-term sustainable and competitive development of the abalone aquaculture industry, genetic improvement techniques have been evaluated and applied around the world, e.g. hybridization in China (Cai et al., 2010, You et al., 2010), Japan (Hoshikawa et al., 1998), Chile (Cruz et al., 2010) and Mexico (Ibarra et al., 2005); ploidy manipulation in Japan (Okumura et al., 2007), Mexico (Maldonado et al., 2001), China (Zhang et al., 1998, 2000) and South Africa (Stepto and Cook, 1998); and

selective breeding in China (Li et al., 2007; Luo et al., 2010; You et al., 2010), Thailand (Amparyup et al., 2010), South Africa (Franchini et al., 2011), New Zealand (Symonds et al., 2012) and Korea (Park and Kim, 2013). In addition, abalone culture has been included in the integrated multi-trophic aquaculture systems to enhance the unit economic gain, such as in South Africa (Nobre et al., 2010) and China (Fang et al., 2009; Troell et al., 2009).

Table 1.1 Abalone aquaculture production (tonnes) from major producing countries and regions.

	2005	2006	2007	2008	2009	2010	2011	2012
China	15460	18921	25324	33010	42373	56511	76786	90694
Chinese Taiwan	9325	10133	5845	7280	3266	2800	2164	2135
Korea	2062	3050	4350	5146	6207	6228	6779	6564
Australia	506	468	504	651	456	455	491	604
Chile	343	395	372	515	843	794	835	828
Mexico	42	45	23	30	47	23	40	64
New Zealand	2	3	8	8	8	80	114	101
South Africa	830	833	783	1037	914	1015	1036	1111
Thailand	20	20	30	30	10	5	5	5
the United States	253	175	175	175	200	250	250	250

Table 1.2 Abalone species farmed in major countries and regions in the world.

China	*Haliotis asinine; H. clathrata; H. diversicolor aquatilis H. diversicolor diversicolor; H. ovina;H. planate; H. varia; H. discus hannai; H. diversicolor*
Chinese Taiwan	*H. discus hannai; H. diversicolor*
Korea	*H. discus; H. discus hannai; H. diversicolor; H. diversicolor supertexta*
Australia	*H. aninina; H. conicopora; H. laevigata; H. roei; H. rubra; H. scalaris*
Chile	*H. discus hannai; H. rufescens*
Mexico	*H. corrugate; H. fulgens; H. rufescens*
New Zealand	*H. australis; H. iris*
South Africa	*H. midae*
Thailand	*H. asinine*
the United States	*H. corrugate; H. fulgens; H. rufescens*

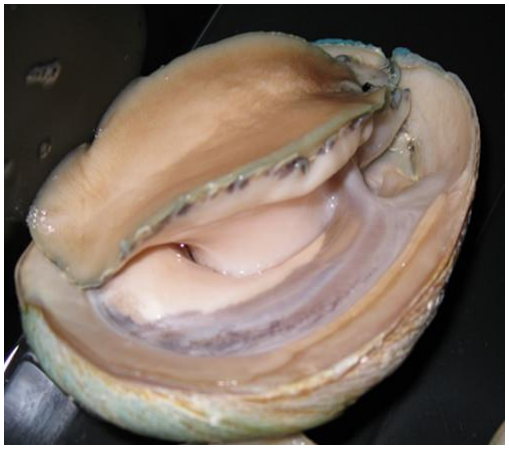
Australian abalone aquaculture started in South Australia (SA) and Tasmania (TAS) in the early 1980s. Since then this industry has been spread to Victoria (VIC) and Western Australia (Table 1.3; Freeman, 2001; Australian fisheries statistics 2012). The species that are cultured or considered for culture in Australia include greenlip *Haliotis laveigata*, blacklip *H. rubra*, brownlip *H. conicopora*, Roe's *H. roei*, donkey ear *H. asinina* and staircase/ridged *H. scalaris* abalone. Among these species, greenlip, blacklip abalone and their hybrid (Fig. 1.1) are the major species currently farmed with advanced culture techniques (Fleming, 1995; Coote et al., 1996; Grove-Jones, 1996; Harris et al., 1997, 1998; Daume et al., 2000, 2004; Grubert and Ritar, 2004, 2005; Grubert et al., 2005; Huchette et al., 2004; Huggett et al., 2005; Freeman et al., 2006; Graham et al., 2006). As with the development of abalone aquaculture overseas, genetic improvement techniques such as (1) hybridization (Hamilton et al., 2009); (2) ploidy manipulation (Norris and Preston, 2003; Liu et al., 2004a, b, 2009; Dunstan et al., 2007; Li et al., 2007); and (3) selective breeding (Selvamani et al., 2001; Baranski et al., 2006, 2008; Lucas et al., 2006; Hayes et al., 2007; Kube et al.,

2007; Li, 2008; Robinson et al., 2010; York et al., 2010) have also been evaluated in Australia. Among these techniques, selective breeding and hybridization have been applied on some farms. However, the efficiency of these programs is compromised due to issues and/or limitations in controlling abalone breeding and biosecurity. For example, Australian government policies restrict the translocation of live abalone between states, leading to the status that selective breeding can be only designed on a state- or farm-basis, which means each state or farm has to establish enough families to allow proper selection to occur. Moreover, although temperature shock in combination with ultraviolet irradiated seawater, or Tris in combination with hydrogen peroxide, has been applied successfully to induce greenlip and blacklip abalone spawning (Liu et al., 2004a; Grubert and Ritar, 2005), these methods cannot ensure that mature males and females spawn synchronously. Consequently, a large number of broodstock are required to spawn each time to achieve the number of families needed in the selective breeding programs and it is difficult to obtain desired mating combinations. Furthermore, blacklip abalone spawn earlier than the greenlip abalone, making the hybridization between these two species even more challenging. Unpredictable natural disasters are another major concern to the abalone aquaculture industry. In 2006, an outbreak of abalone viral ganglioneurits in Victoria destroyed a selective breeding nucleus after more than 7 years' of efforts. In livestock and finfish species, sperm cryopreservation has been proven to be a reliable and effective technique which could help solve these issues facing abalone genetic improvement programs in Australia (Purdy 2006; Tiersch 2008; Mocé and Vicente 2009; Cabrita et al. 2010).

Table 1.3 Abalone aquaculture production (tonnes) and value ($000) in Australia. The symbol "-" means missing values.

	Production				**Value**			
	SA	VIC	TAS	AUS	SA	VIC	TAS	AUS
2000 - 2001	53	5	-	58	2677	232	-	2909
2001 - 2002	34	13	-	47	1901	631	-	2532
2002 - 2003	27	14	-	41	907	560	-	1467
2003 - 2004	105	102	43	250	3155	3561	1625	8341
2004 - 2005	177	124	89	390	5318	4454	3384	13156
2005 - 2006	250	161	95	506	8222	5633	3753	17608
2006 - 2007	196	121	152	468	7155	4344	5990	17489
2007 - 2008	167	166	171	504	5151	5964	5803	16919
2008 - 2009	227	179	245	651	8121	6623	8312	23056
2009 - 2010	286	-	170	455	10341	-	5099	15440
2010 - 2011	317	-	173	491	10842	-	5547	16389
2011 - 2012	178	330	97	604	6410	9681	3101	19192

SA: South Australia; VIC: Victoria; TAS: Tasmania; AUS: Australia.



Greenlip abalone

Haliotis laevigata Donovan

Blacklip abalone

Haliotis rubra Leach

(Photoed by SARDI)

Hybrid (greenlip x blacklip) abalone

(Photoed by Great Southern Waters)

Figure 1-1 Farmed major abalone type in Australia.

Chapter 1. General introduction

The milestone for sperm cryopreservation dates back to 65 years ago when the cryoprotective property of glycerol was discovered by Polge et al. (1949). Since then, sperm cryopreservation has developed dramatically and protocols have been optimized for many species (Janett et al., 2003; Bhakat et al., 2011) and applied for various purposes, such as livestock and finfish breeding (Purdy, 2006; Mocé and Vicente, 2009; Cabrita et al., 2010; Rodriguez-Martinez and Wallgren, 2011), human reproduction (Stanic et al., 2000), and endangered species conservation (Medeiros et al., 2002; Benson et al., 2012).

During cryopreservation, sperm generally experience the processes of temperature reduction, osmotic change, freezing and thawing. Cellular damage can be caused by each of these processes and the post-thaw sperm quality is dependent on the combined effects of these injuries (Medeiros et al., 2002). In order to optimize a sperm cryopreservation technique, it is essential to have a good understanding of the key processes causing cryoinjuries.

The temperature reduction during cryopreservation includes cooling and freezing steps. The cooling step has an irreversible influence, known as cold shock, on sperm metabolism, membrane permeability, motility and biochemical components when the temperature is decreased from normothermia to 0 ~ 4 ºC (Drobnis et al., 1993; Zeng and Terada, 2001; Medeiros et al., 2002; Pasqualotto et al., 2012). The severity caused by cold shock is species-specific in mammals as sperm from different species have different sensitivity against cold shock, e.g. rabbits, humans and roosters > dogs and cats > cattles, rams and stallions > boars (Zeng and Terada, 2001; Medeiros et al., 2002; Mocé and Vicente, 2009). Reasons for this phenomenon may be that human and rabbit sperm have a higher cholesterol/phospholipid ratio and a lower polyunsaturated/saturated fatty acid ratio in phospholipids than other animals (Mocé

and Vicente, 2009). However, cold shock has little impact on sperm in aquatic species as normally, the collected sperm are directly placed at a low temperature of 0 ~ 4 ºC without the addition of any cryoprotectant agents. The possile reasons underlying this phenomenon are that the aquatic species are poikilothermic and their sperm characteristics adapt more readily to temperature reduction than in homoeothermic species (Bobe and Labbe, 2009).

In the freezing step, various cooling rates have been evaluated for sperm cryopreservation in human, livestock and aquatic species. Slow cooling rates can concentrate the intracellular solutes by gradually removing water from cells, thus inducing cellular dehydration and reducing the chance of intracellular ice formation. At the same time, the slow cooling can also cause severe cellular volume shrinkage and a long-term exposure to a high concentration of solute (known as solute effects), leading to sperm injuries. Fast cooling rates, on the other hand, can reduce the solute effects to certain extend as less water will be removed from the sperm. As a consequent of relatively higher water concentration, large intracellular ice crystals can form, causing physical damage to sperm organelles and components (Gao and Critser, 2000; Holt, 2000; Lemma, 2011). The optimal cooling rate should balance all these factors. A plot presenting the interaction between freezing rate solute toxic injury, ice formation injury and sperm survival has been drawn by Muldrew et al. (2004), which show an inverted “U” shaped curve. Both programmable and non-programmable freezing methods can be used to deliver the freezing rate required during cryopreservation. Programmable freezing method utilize specialized equipment which is able to give tight control over the freezing rate (Stanic et al., 2000; Rota et al., 2005; Clulow et al., 2008; Hopkins and Herr, 2010). Non-programmable freezing method normall utilize a modified styrofoam box containing

a rack. The method can only control the end point temperature by adjusting the rack height above the liquid nitrogen surface (Ieropoli et al., 2004; Dong et al., 2005b, c, 2006).

The cryoprotectant agent (CPA) is essential for sperm cryopreservation and is usually divided into permeable and non-permeable categories according to their ability to penetrate the sperm membrane. The permeable CPAs act intracellularly and extracellularly and help minimize intracellular ice formation, rearranging the membrane lipids and proteins, and increasing the likelihood for sperm to survive cryopreservation (Holt, 2000; Benson et al., 2012). Different permeable CPAs, such as dimethyl sulfoxide (DMSO), glycerol, ethylene glycol, propylene glycerol and methanol, are used for sperm cryopreservation in human, livestock and aquatic species. The non-permeable CPAs act extracellularly and have the ability to stabilize the sperm membrane to manage the influx and efflux of solute during cryopreservation (Holt, 2000). Different non-permeable CPAs, such as egg yolk, sugars, skim milk, macromolecules, amino acids and vitamins, have been evaluated in combination with the permeable CPAs to reduce cryoinjuries. A proper CPA should impose minimal impacts of osmotic pressure change and toxicity when the sperm are exposed to CPA addition before freezing and removal after thawing (Hammerstedt and Graham, 1992; Denniston et al., 2000; Salinas-Flores et al., 2005).

Thawing methodology is dependent on the cooling rate in a given system, sperm type and surface area to volume ratio of the cryo-container used (Gao and Critser, 2000; Holt, 2000). The thawing step can form a harmful large ice crystal from small intracellular ice crystals if a rapid cooling rate is used. Moreover, the thawing step can cause excessive osmotic swelling during the CPA removal. Thawing temperatures from 5 to 80 ºC have been used in human, livestock and finfish sperm

cryopreservation (Suquet et al., 2000; Purdy, 2006; Mocé and Vicente, 2009).

Apart from the cryopreservation processes, broodstock physiological conditions, such as the environment where the broodstock live, broodstock age and the time when sperm are collected over a natural spawning season, are also important as these factors affect the initial sperm quality, the prerequisite for the success of sperm cryopreservation. Differences in initial sperm characteristics exist between sperm from wild and farmed cod *Gadus morhua* (Skjæraasen et al., 2009) and Chinook salmon *Oncorhynchus tshawytscha* (Lehnert et al., 2012). These differences affect sperm cryosensitivity and need to be accounted for when developing suitable sperm cryopreservation protocols (Martinez-Pastor et al., 2005; Flores et al., 2008; Muiño et al., 2009; Ortega-Ferrusola et al., 2009; Beirão et al., 2011).

Development of methods for effectively and efficiently measuring sperm quality at subcellular levels has been a recent focus, revealing sperm cryoinjuries in human, livestock and finfish species, helping to develop strategies to minimize the cryoinjuries and optimizing cryopreservation protocols (El-Sheshtawy et al., 2008; Khalili et al., 2010; Martínez-Páramo et al., 2012; Memon et al., 2013) as the integrity of organelles/subcomponents are pivotal for successful fertilization. For example, integral plasma membrane plays an important role in regulating cellular exchange, functional mitochondria generate energy required to drive the sperm toward the oocyte and an intact acrosome is necessary for releasing the enzymes required to allowfertilization. The assessment of cryoinjuries to these organelles has been enhanced with the introduction of specific fluorescent dyes (Thomas et al., 1997; He and Woods, 2004; Salinas-Flores et al., 2005; Smith et al., 2012b). Recently, flow cytometry has been employed to improve the accuracy and efficiency of sperm quality assessment techniques using fluorescent staining as a large number

of sperm can be evaluated within a very short period (Salinas-Flores et al., 2005; Paniagua-Chávez et al., 2006; Cabrita et al., 2010).

A non-programmable sperm freezing technique has been developed in greenlip abalone using sperm collected from wild broodstock (Zhu et al., 2014). However, when this protocol is used in farmed counterparts, highly variable and low fertilization rates have been produced, hindering the application of sperm cryopreservation technique to improve the efficiency of abalone genetic improvement programs and preserve the superior genetics in Australia. Therefore, this PhD project was established by the Australian Seafood Corporative Research Centre with the support from the Australian Abalone Growers Association. The overall aims of this project are to understand the reasons causing the discrepancies in cryopreservation success between wild and farmed abalone and to develop a sperm cryopreservation protocol suitable for farmed abalone species in Australia. After the literature review and consultations with industry the following objectives are developed to achieve the overall project aims:

1) To investigate differences in cryo-sensitivities between sperm collected from farmed and wild abalone;

2) To investigate the effects of key physiological conditions on pre- and post-cryopreservation sperm quality in farmed abalone;

3) To improve the efficiency and accuracy of abalone sperm quality assessment methods to determine the integrity of sperm components and/or functionality of organelles;

4) To investigate methods to mitigate cryoinjuries to sperm in farmed abalone;

5) To develop a sperm cryopreservation technique suitable for farmed abalone in Australia.

The thesis consists of 8 chapters. This Chapter reviews the current status of abalone aquaculture in the world, and justifies the need for the research presented and the objectives of this PhD thesis. Chapters 2 ~ 7 were designed to achieve the project objectives and were presented as unabridged versions of the manuscripts published or under review in peer-reviewed journals. Athough some co-authors are included in each manuscript for publication, the thesis research was initiated, implemented and completed by the PhD candidature. For consistency, all chapters have been re-formatted in this thesis though differnet formats were used in published papers.

Chapter 2 systematically reviewed a) the information on marine mollusc sperm cryopreservation in published studies, b) recent developments in cryoinjury investigation at subcellular levels, c) factors influencing sperm cryosensitivity, and d) cryoinjury mitigation techniques developed. Based on these information and consultation with abalone aquaculture industry, the experiments were designed to achieve the thesis objectives.

In Chapter 3, equipment that can control the set temperature and heat exchange better than those used in the study by Zhu et al. (2014) was applied to improve the non-programmable freezing technique in greenlip abalone and investigated the differences in cryosensitivity between sperm collected from wild and farmed abalone (Objective 1). First, the 0.25 mL straw was used in this study to improve the heat exchange efficiency during freezing and thawing as this straw had a much higher surface to volume ratio than the 2.0 mL cryovial used in a previous study (Zhu et al., 2014). Second, the floating rack was employed in this study to improve the accuracy of end point temperatures as the distance between the rack and the liquid nitrogen

surface was less affected by the liquid nitrogen evaporation in this method than the fixed rack method (Zhu et al., 2014). The experiments also included the assessment of the relationship between sperm motility/fertilization rates and integrity of component and/or functionality of organelles revealed by fluorescent staining (Objective 3).

Chapter 4 investigated the influence of broodstock age and sperm collection time over a natural spawning season on sperm cryosensitivity in farmed greenlip abalone to identify broodstock physiological conditions suitable for sperm cryopreservation (Objectives 2 and 5). Moreover, flow cytometry was used to differentiate sperm of varying cryosensitivity from broodstock experiencing different physiological conditions to improve the efficiency of fluorescent staining sperm quality assessment (Objective 3). This technique was then used to assess the occurrence of cryoinjuries in subsequent experiments.

Chapter 5 investigated the application of programmable freezing technique to farmed abalone as this method produced better and more consistent results than the non-programmable technique in sperm cryopreservation on other species (Objectives 4 and 5). Parameters optimized in Chapter 4, such as broodstock age and sperm collection time over a natural spawning season, were adopted.

As low post-thaw sperm quality was revealed using the programmable freezing technique in the previous chapter, Chapter 6 was designed to further improve the non-programmable freezing technique by evaluating additional cryoprotective agents, vitamin (L-ascorbic acid), amino acids (glycine and taurine), and monosaccharides (glucose, fructose and galactose) (Objectives 4 and 5).

Chapter 7 tested the non-programmable freezing technique on sperm from farmed

blacklip abalone, another economic species farmed in Australia. In addition, sperm from blacklip abalone were also used to produce hybrids, which are preferred for production by some abalone farms in Victoria and Tasmania (Objective 5).

The general discussion in Chapter 8 integrated the results from all chapters of this thesis in an attempt to comprehend the research findings and advance the existing understanding and knowledge in this research field. Based on the conclusions from this thesis, further research directions were proposed to tackle some outstanding questions.

CHAPTER 2 : SPERM CRYOPRESERVATION IN MARINE MOLLUSC: A REVIEW*

* This chapter has been submitted as a stand alone manuscript to *Aquaculture International* as: **Liu, Y.**, Li, X., Robinson, N., Qin, J., Sperm cryopreservation in marine mollusc: a review (under review). Manuscript Number: AQUI-D-14-00381.

2.1 Abstract

Sperm cryopreservation is a valuable technique that can enhance the efficiency of genetic improvement programs, commercial production and endangered species conservation. Although studies on sperm cryopreservation in marine molluscs started late in comparison with livestock and finfish, research has substantially increased for the major economical species farmed in the world, including oysters, mussels, scallops, pearl oysters, clams and abalone. In this paper, the latest developments in marine bivalves and gastropods sperm cryopreservation were reviewed and summarized for seven key steps: 1) sperm collection, 2) extender selection, 3) cryoprotectant agent selection, 4) sperm dilution/concentration before cryopreservation, 5) cooling rate, 6) thawing temperature, and 7) quality assessment.

In general, the sperm quality is controlled by physiological and environmental parameters and further compromised by the cryopreservation processes. However, less attention has been paid to these issues in sperm cryopreservation in marine bivalves and gastropods. Discussion about factors affecting sperm quality is, therefore, extended to include information from livestock and finfish species where they have been largely evaluated. Knowledge on these factors would not only improve our understanding of the key parameters influencing the success of sperm cryopreservation, but also provide information to develop strategies to improve the sperm quality in marine molluscs.

Keywords: marine mollusc, sperm cryopreservation, sperm quality, improvement

2.2 Introduction

Mollusc aquaculture has rapidly developed over the last 60 years with recent production reaching 15.2 million metric tons and worth 15.9 billion USD in 2012 (FAO, 2012). Mollusc account for about 23% by weight and 11% by value of the total world aquaculture production, and above 98% is from marine species, such as oysters, clams, mussels, abalone and scallops (FAO, 2012). Genetic improvement techniques, such as selective breeding and hybridization, have been evaluated and initiated in some species to improve the production and ensure the long-term sustainable and competitive development of the industry (Li, 2008). The potential for genetic improvement will be further enhanced if: (1) spawning could be synchronized between males and females within and between species in a breeding season; (2) the short window period of natural spawning could be extended; (3) restrictions on transportation of live animals between states or countries could be reduced and (4) risks associated with keeping superior broodstock alive and healthy could be reduced.

Sperm cryopreservation is a proven technique for long-term storage using liquid nitrogen (LN) as a coolant and offers opportunities to address the above issues by (1) providing sperm without seasonal limitations; (2) providing a comparatively safe repository for improved lines with valuable and desired traits to minimize the risk due to disease outbreaks and natural disasters; and (3) facilitating dissemination of superior genetic materials between locations with disease issues minimized (Adams et al., 2004; Dong et al., 2005b, c, 2006, 2007a; Salinas-Flores et al., 2005; Matteo et al., 2009; Zhang et al., 2012).

Sperm cryopreservation techniques have been extensively developed in livestock

species and have become a near billion-dollar business globally (Tiersch, 2008; Mocé and Vicente, 2009). For example, cryopreservation has been routinely applied to assist horse (Janett et al., 2003) and cattle breeding programs (Bhakat et al., 2011). In aquatic species, sperm cryopreservation research has been largely investigated in finfish to solve the similar issues, with about 200 species having been cryopreserved (Cabrita et al., 2010). Studies on marine mollusc sperm cryopreservation have increased over the last decade, especially for oysters (Dong et al., 2005b, c, 2006, 2007a; Adams et al., 2008; Zhang et al., 2012). In general, sperm cryopreservation includes sperm quality assessment, sperm collection, extender preparation, cryoprotectant agent (CPA) preparation, cooling, storage, thawing and post-thaw sperm evaluation. Research in marine mollusc has mainly focused on the selection of extender, CPA, cooling rate and thawing temperature using sperm motility, ultrastructure, morphology, fertilization rate and integrity of components and organelles as quality assessment indicators (Adams et al., 2003; Gwo et al., 2003; Kang et al., 2004; Dong et al., 2005b; Salinas-Flores et al., 2005; Smith et al., 2012a, b; Zhang et al., 2012; Liu et al., 2014a, b, in press b). Other information, such as environmental and physiological factors, has not yet to be evaluated. However, these factors have been found to affect sperm quality in livestock and finfish species which further influence the sperm ability to tolerate cryopreservation (Lanes et al., 2010; Lymberopoulos et al., 2010). Understanding these factors will provide effective information to optimize and/or improve the reliability of sperm cryopreservation techniques in marine mollusc. Our recent findings have shown that knowledge of factors influencing and improving sperm quality in livestock and finfish species can be helpful and this knowledge has been developed and applied to greenlip abalone *Haliotis laevigata* (Liu et al., 2014a, b) and blacklip abalone *H. rubra* (Liu et al., in

press b). Furthermore, equipment developed for sperm cryopreservation in livestock species is applicable to oysters (Dong et al., 2007a). In this paper, we (1) critically review the latest advances in sperm cryopreservation of economically important marine gastropod and bivalve species; and (2) discuss factors known to affect sperm quality and suggest strategies to improve marine mollusc sperm cryopreservation, by including relevant knowledge from studies of livestock and finfish species.

2.3 Cryopreservation

2.3.1 Sperm collection method

Two methods are normally used to collect sperm from marine mollusc: natural spawning and strip spawning. The former method is often used in mussels, abalone and scallops with temperature shock being the most common stimulus. Other treatments, such as desiccation and ultraviolet irradiation, coupled with temperature shock are also used to induce spawning in abalone and scallops (Table 2.1). With natural spawning, sperm quality is vulnerable to contamination by mucus, seawater or faeces and it is also not easy to collect the concentrated sperm (Gwo et al., 2002). Centrifugation has been evaluated as a way of boosting the concentration of sperm collected from scallops *Patinopecten yesoensis* (Yang et al., 2007) and abalone (Gwo et al., 2002; Salinas-Flores et al., 2005). However concentration with centrifugation has not produced high post-thaw sperm fertilization rates when applied to abalone cryopreservation (less than 60%) (Gwo et al., 2002; Salinas-Flores et al., 2005). Dry spawning (natural spawning without seawater) is an alternative way to collect concentrated sperm and can help avoid contamination caused by seawater, mucus or faeces in natural spawning. This technique has been applied to greenshell mussels *Perna canaliculus* (Smith et al., 2012a), greenlip (Liu et al., 2014a, b) and blacklip

abalone (Liu et al. in press b) with above 80% post-thaw sperm fertilization rates achieved.

Strip spawning has been routinely applied to oysters and pearl oysters. There is one report of this method being used for disc abalone *H. discus hannai* (Kang et al., 2004), whereas other researchers have had little success when strip spawning is used for small abalone *H. diversicolor* (Gwo et al., 2002), greenlip (Liu et al., 2014a, b) or blacklip abalone (Liu et al., in press b). Also, this method is not recommended for scallop *P. yesoensis* (Yang et al., 2007). Our recent study has found that mussel *M. galloprovincialis* sperm can be cryopreserved after collection using strip spawning and that about 90% post-thaw sperm fertilization rates could be achieved (relative to control) (unpublished data). Using this sperm collection method, variations in fresh sperm motility for *M. galloprovincialis* were observed, which may be attributed to the differences in the stage of spermatogenesis in individuals. Variation in fresh sperm motility has been observed in the blacklip pearl oysters *Pinctada margaritifera* (Acosta-Salmón et al., 2007) and Eastern oysters *Crassostrea virginica* (Yang et al., 2012) which may be considered to influence the post-thaw results (Acosta-Salmón et al., 2007; Yang et al., 2012). However, factors affecting fresh sperm quality and the correlation with post-thaw sperm quality are not known.

A new method has been reported for Macha surf clam *Mesodesma donacium* by Dupré and Guerrero (2011), where pieces of gonad were dissected and directly cryopreserved. This method resulted in 97% post-thaw fertilization rates which were two times higher than for sperm collected using a strip spawning method. The high post-thaw fertilization rate may be because components in the seminal plasma from the gonad, such as antioxidants, exist at optimal concentrations that are difficult to

replicate artificially and more sperm obtained from testicular pieces (Dupré and Guerrero, 2011).

2.3.2 Extender

In marine mollusc, the selection of an appropriate extender may be dependent on the sperm collection method. With strip spawning, calcium-free Hanks' balanced salt solution is often used (Table 2.1). This extender may be beneficial for sperm motility, fertilizing ability and larval production (Paniagua-Chávez et al., 1998) and is routinely used in Pacific oysters *C. gigas* (Dong et al., 2005b, c, 2006 2007a) and Eastern oysters (Paniagua-Chávez and Tiersch, 2001; Paniagua-Chávez et al., 2006). Reasons for use of a balanced salt solution may be due to its ability to inhibit sperm activation which is considered to help conserve energy in the sperm before freezing (Dong et al., 2005b). Osmolarity of extender is also an important factor for sperm collected by strip spawning (Dong et al., 2005b). Extenders with sub-optimum osmolarity have detrimental effects on sperm cryopreservation for Eastern oysters (Paniagua-Chávez et al., 1998) and Pacific oysters (Dong et al., 2005b, 2007a). Inhibition of sperm activation and optimization of osmolarity for sperm could be beneficial for maintaining the sperm quality during cryopreservation using strip spawning method.

Natural seawater is the most frequent extender used when sperm is collected after natural spawning in mussels, abalone, scallops and clams (Table 2.1). Artificial seawater is also used, but has only been applied to small abalone (Gwo et al., 2002; Kang et al., 2004). This method does not inhibit sperm activation as sperm are already activated after natural spawning. Sperm quality, such as fertilizing ability, deteriorates with longer periods of suspension in extender in small abalone (Gwo et

al., 2002) and mussels *Mytilus galloprovincialis* (Matteo et al., 2009), although biological ability of the sperm can be prolonged at lower temperatures. Understanding the mechanisms of sperm motility activation, and discovering the extender with the ability to minimize or inhibit sperm motility after natural spawning, may be beneficial for conserving metabolic energy and prolonging biological ability of the sperm, resulting in higher sperm quality after cryopreservation.

2.3.3 Cryoprotectant agent

The ability of the cryoprotectant agent (CPA) to penetrate the sperm membrane can be categorized as permeable and non-permeable (Dong et al., 2005b). In this review, we refer to the permeable CPA as CPA, and non-permeable CPA as co-CPA. Dimethyl sulfoxide (DMSO), glycerol, ethylene glycol (EG), propylene glycerol (PG) and methanol have been used as CPAs. Among these agents, DMSO is frequently used at concentrations between 5 and 20% in oysters, pearl oysters, mussels, abalone, scallops and clams (Table 2.1). However, some of these studies have selected specific CPA directly without comparison to other types and concentrations of agent (Table 2.1). Co-CPA has been found to improve cryosurvival as it is able to stabilize the sperm membrane by controlling the influx and efflux of solute during cryopreservation (Dong et al., 2006). Co-CPAs, such as trehalose, polyethylene glycol (PEG), glycine and sucrose, can be used in combination with or without CPAs (Table 2.1).

In Pacific oysters, DMSO, methanol and EG are used more often than other CPAs and PEG has been used as co-CPA in combination with DMSO or methanol (Adams et al., 2004, 2008; Ieropoli et al., 2004; Dong et al., 2007a). Moreover, glycine has been preferentially selected as a cryoprotective medium in some studies (Yankson

and Moyse, 1991; Zhang et al., 2012). Reasons leading to the selection of different CPAs are unknown, but could be due to genetic variation in sperm characteristics as broodstock were sourced from different geographic locations (Thurston et al., 2002; Ieropoli et al., 2004; Dong et al., 2005c).

In Eastern oysters (also named American oysters), only PG and DMSO have been evaluated (Paniagua-Chávez and Tiersch, 2001; Paniagua-Chávez et al., 2006). In scallops (*Argopecten purpuratus* and *Chlamys farreri*) and Macha surf clam, 10 ~ 16% DMSO is frequently used as a CPA, and about 5% sucrose and 10% yolk have been added as co-CPAs (Li et al., 2000; Espinoza et al., 2010; Dupré and Guerrero, 2011).

Methanol and DMSO with different co-CPAs are used for sperm cryopreservation in Japanese pearl oysters *P. fucata martensii* and blacklip pearl oysters, respectively (Lyons et al., 2005; Acosta-Salmón et al., 2007; Kawamoto et al., 2007; Narita et al., 2008a, b; Hui et al., 2011). The addition of fetal bovine serum plays a positive role in the post-thaw sperm motility of Japanese pearl oysters which may be due to its ability to reduce osmotic and ionic effects and enhance cryoprotective effects during freezing (Kawamoto et al., 2007). The addition of sugars, such as glucose, sucrose or trehalose, has been found not to improve the post-thaw sperm motility and to significantly lower motility with increased sugar concentrations (Kawamoto et al., 2007). In contrast, trehalose plays an important role in blacklip pearl oysters. Acosta-Salmón et al. (2007) has demonstrated that the use of trehalose alone achieved similar post-thaw sperm motility as it does in combination with DMSO. Likewise, Lyons et al. (2005) has found that the addition of trehalose significantly increased post-thaw sperm motility.

In abalone, DMSO and glycerol at concentrations of 6 ~ 10% and 5 ~ 10% have been

used, respectively (Table 2.1), with the former producing better results (Table 2.2). For sperm cryopreservation in new abalone species, DMSO and glycerol should be evaluated first, as other CPAs used in marine mollusc, such as EG and PG, have not been selected in all the published studies so far. Regarding co-CPAs, sugars, amino acids and vitamins were not used by published studies (Table 2.1). Nevertheless, in our recent studies, the addition of 1 or 2% glucose significantly improved the post-thaw sperm fertilization rates in greenlip and blacklip abalone, respectively (Liu et al., 2014b, in press b). In addition, further addition of 0.6% glycine could also significantly improve the post-thaw sperm fertilization rates in farmed greenlip abalone (Liu et al., 2014a). These results and those from Pacific oyster studies indicate that glycine play a positive role in sperm cryopreservation in marine mollusc.

2.3.4 Sperm dilution/concentration before cryopreservation

Dilution is of importance to the success of sperm cryopreservation as less sperm dilution may cause detrimental agglutination after thawing and higher dilution may cause a rapid depletion of energy, changes to physiology and reduced quantities of the amount of protective components in seminal plasma, resulting in the reduced viability (Paniagua-Chávez et al., 1998; Dong et al., 2005b). The dilution of sperm inside the cryo-container needs to balance the above effects and achieve the highest fertilizing ability using the least number of cryopreserved sperm. In general, sperm collected from natural spawning can be diluted the least, at a ratio of up to 1:10, while those collected from strip spawning can be diluted more, at a ratio from 1:8 to 1:20 (Table 2.1), though higher ratios (from 1:49 to 1:100) have been applied to Japanese pearl oysters (Narita et al., 2008a, b) and *Ostrea edulis* (Vitiello et al., 2011). Reasons for higher dilution with strip spawning may be due to the higher concentration of collected sperm. Compared with dilution ratios, diluting sperm to

specific concentrations would be more informative for protocol comparison and optimization, such as 1×10^9 mL^{-1} for Pacific oysters (Dong et al., 2005b, c, 2006, 2007a), and 1.6×10^8 mL^{-1} for greenlip and blacklip abalone (Liu et al., 2014b, in press b).

2.3.5 Cooling rate

Techniques to freeze sperm in marine mollusc can be categorized as non-programmable (LN vapor) and programmable. A balance between the increased solute concentration and the occurrence of intracellular ice formation is important and necessary in both freezing techniques. The non-programmable freezing technique can process a larger number of straws with low cost in equipment and is easy to operate on-site, normally using styrofoam boxs with racks. This technique has produced more than 75% post-thaw sperm fertilization rates in oysters, pearl oysters, mussels, abalone and scallops (Table 2.2). With non-programmable freezing techniques, the samples are placed at a level of 3 ~ 25 cm above the LN surface for 3 ~ 20 min (Table 2.2) or at a pre-freezing temperatures of -30 to -120°C adjusted by changing the distance from the LN surface (Gwo et al., 2002; 2003; Lyons et al., 2005; Narita et al., 2008a, b; Zhu et al., 2014). Recording the temperature changes when exposure to LN vapor may help better understand the cooling rate effects on sperm cryopreservation.

Programmable freezing requires special equipment to control the cooling rate(s). The samples are usually cooled and frozen according to the cooling rate periods with or without a holding period in between prior to transfer into LN for long-term storage. In the protocols with one cooling period, a higher endpoint temperature of -30°C with a slow cooling rate of -2.5 °C/min (Paniagua-Chávez and Tiersch, 2001;

Paniagua-Chávez et al., 2006), or a lower endpoint temperature of -70 to -100°C with either slow (-3, -6 or -8 °C/min) (Ieropoli et al., 2004; Espinoza et al., 2010; Vitiello et al., 2011) or fast (-18 to -50 °C/min) cooling rates (Li et al., 2000; Kang et al., 2004; Dupré and Guerrero, 2011) have been used. In the protocols with two cooling periods, cooling rates in the first period are slower than the second (Dong et al., 2005b, 2006). So far, only one study in mussels has used three cooling periods (Matteo et al., 2009).

A dry ice/methanol bath is used in some studies. Samples are placed in a bath for 10 min before being plunged into LN. This technique resulted in 80 ~ 90% post-thaw sperm fertilization rates in Pacific oysters (Adams et al., 2004, 2008). Vitrification has also been evaluated in marine mollusc, but highly variable and poor results have been obtained which may not be used (Adams et al., 2004; Kang et al., 2004; Wang et al., 2006; Acosta-Salmón et al., 2007).

The commercial dairy bull sperm freezing system has been evaluated for Pacific oyster sperm cryopreservation (Dong et al., 2005b). Use of this system could lower the initial capital investment, reduce the economic risk and store hundreds of straws, and therefore be beneficial for the oyster aquaculture industry. Results from this evaluation indicated that the existing sperm cryopreservation protocols from livestock species may be applicable to marine mollusc. Moreover, the non-programmable freezing technique has also been used in Pacific oysters to assist breeding programs and has achieved acceptable post-thaw sperm fertilization rates (Adams et al., 2008). In other species, non-programmable freezing techniques produce either similar to or better than the results of using programmable freezing techniques (Table 2.2), suggesting that the former should be considered as the first

choice for the development of sperm cryopreservation in new marine mollusc species.

2.3.6 Thawing temperature

In marine mollusc, thawing temperature can be categorized into three ranges: low (< 29°C), medium (30 ~ 49°C) and high (> 50°C) (Table 2.2). In Pacific oysters, low, medium and high ranges have all been applied resulting in a 80% post-thaw sperm fertilization rate except at 70°C. A similar trend was found in abalone (Table 2.2). In contrast, only the low range (18 ~ 25°C) was used in pearl oysters and mussels. In scallops, low (22 ~ 26°C) *P. yesoensis* (Yang et al., 2007) and high (50°C) *A. purpuratus* (Espinoza et al., 2010) ranges were used with the last achieving the highest post-thaw sperm fertilization rates. However, theory enabling the selection of appropriate thawing temperatures for marine mollusc sperm cryopreservation is lacking.

2.3.7 Sperm quality assessment

The current parameters to assess sperm quality in marine mollusc include motility rate, fertilization rate, morphological change and organelle integrity. Motility can be measured directly by counting the percentage of motile sperm in a subsample (Lyons et al., 2005; Vitiello et al., 2011). Moreover, sperm motility can also be measured by computer assisted sperm analysis (CASA) systems which are able to assess a large number of sperm and distinguish motility patterns, giving higher accuracy and efficiency than subjective observation (Acosta-Salmon et al., 2007). Although the CASA system has been used in some species in marine mollusc, no studies have characterized the sperm subpopulations in terms of movement patterns. Identification of different sperm subpopulations may be beneficial for understanding and predicting the cryopreservation effects as different subpopulations have been found to have

different abilities to tolerate cryodamage (Thurston et al., 2002). Differences in fertilizing ability and freezability have been found between subpopulations of sperm in boar and donkey (Flores et al., 2008), cattle (Muiño et al., 2009) and gilthead seabream *Sparus aurata* (Beirão et al., 2011). Furthermore, computer-assisted morphometry analysis (CAMA) techniques can further discriminate morphometric characteristics within sperm subpopulations, which in turn provide useful information to understand the ability to tolerate cryopreservation processes with morphological changes. This technique has been applied to goats (Gravance et al., 1995), rams (Gravance et al., 1998), European eel (Asturiano et al., 2007) and deer (Esteso et al., 2009), but not to marine mollusc.

Fertilization measures the sperm ability to produce viable embryos and the sperm to egg ratio is critical for successful measurement. Both lower and higher ratios have been found to affect the fertilization rate (Adams et al., 2008; Narita et al., 2008b). With strip spawning, sperm to egg ratios above 5000 are often used, whereas ratios below 5000 are often used for natural spawning. This difference could be because sperm exists at a different stage of spermatogenesis with strip spawning, resulting in different responses to activation. In Pacific oysters, although a higher sperm to egg ratio of 5000-10000:1 is commonly used, lower ratios of 10 ~ 15:1 have also produced results similar to controls (Ieropoli et al., 2004). In scallops (*C. farreri*), sperm to egg ratios of 3 ~ 15:1 have resulted in higher fertilization rates than ratios of 500 ~ 1000:1 (Yang et al., 1999; Li et al., 2000; Yang et al., 2007). In abalone, ratios of 3500 ~ 4000:1 resulted in better outcomes than other ratios assessed (Zhu et al., 2014). In mussels, both higher and lower sperm to egg ratios have been evaluated and both resulted in significantly lower post-thaw sperm fertilization rates than controls (Matteo et al., 2009; Smith et al., 2012a).

When undertaking morphological evaluations for sperm, observations are traditionally made of changes to the sperm head, middle piece, tail, acrosome, mitochondria and nucleus by scanning and/or transmission electron microscopes. These observations provide effective information for understanding the effects of cryopreservation on sperm (Espinoza et al., 2010). Nevertheless, these assessments are slow to perform. A better balance between the evaluation period, and the short window period for natural spawning in marine mollusc is necessary and important.

Flow cytometry is another technique used to assess sperm quality, and it has the potential for evaluating multiple sperm parameters in a short period. This technique has been used for red abalone *H. rufescens* (Salinas-Flores et al., 2005), Eastern oysters (Paniagua-Chávez et al., 2006) and greenlip abalone (Liu et al., 2014a) with fluorescent dyes, such as SYBR14/ propidium iodide (PI) for plasma membrane integrity, rhodamine 123/PI for mitochondrial membrane potential and LysoTracker green DND-26/PI for acrosome integrity. In addition, the fluorescent dyes can be observed under a fluorescent microscope which makes the technique more accessible than flow cytometry (Smith et al., 2012b; Liu et al., 2014b).

Table 2.1 Sperm collection method, extender, cryoprotectant agent (CPA) or CPA/co-CPA combination and dilution/concentration used in marine mollusc sperm cryopreservation.

Species	**collection methods**	**extenders**	**CPA(s) or CPA/co-CPA(s) combination**	**dilution/ concentration**	**references**
C. gigas	strip spawning (SP)	artificial seawater (ASW)	10% DMSO		Gwo et al., 2003
		SW	10% EG	1:10	Ieropoli et al., 2004
			5% DMSO + 0.45M TRE	1:10	Adams et al., 2004
			5% DMSO + 0.54M TRE	1:9	Adams et al., 2008
		Calcium-free Hanks' balanced salt solution (C-F HBSS) 1000	8% DMSO	1:1 1×10^9/ml	Dong et al., 2005b
			6% MET		Dong et al., 2005c
			6% PEG with 4% PG or 4% DMSO		Dong et al., 2006
			5 or 6% MET; 4% MET + 2% PEG		Dong et al., 2007a
		SW	6% DMSO + 0.6% glycine	6.25×10^7/mL	Zhang et al., 2012
C. virginica	SP	C-F HBSS	10 or 15% PG	1:1	Paniagua-Chavez and Tiersch, 2001
		C–F HBSS 600	5 or 10% PG		Paniagua-Chávez et al., 2006
O. edulis		SW	15% EG	1:100	Vitiello et al., 2011
P. fucata martensii		80% SW	10% MET + 20% FBS	1:49 $3*10^8$/ml	Kawamoto et al., 2007
					Narita et al., 2008a
					Narita et al., 2008b
P. marganitifera		C-F HBSS	5% DMSO + 1M TRE	1:12.5	Lyons et al., 2005
			0.45M TRE + 0M, 0.64M, 1.02M or 1.53M DMSO	1:10	Acosta-Salmón et al., 2007
			0.8M DMSO + 0.7M TRE	1:10	Hui et al., 2011

P. martensii		SW	10% DMSO	1:20	Wang et al., 2006
M. galloprovincialis	NP by temperature shock (TS)		7% EG	1:10	Matteo et al., 2009
P. canaliculus			12% DMSO + 0.2M TRE	1:1 or 1:3	Smith et al., 2012a
H. discus hannai	SP	ASW 1200	5% GLY	1:9	Kang et al., 2004
H. diversicolor	NP by air exposure + TS	SW	8% DMSO		Tsai and Chao, 1994
		ASW 1100	10% DMSO		Gwo et al., 2002
H. laevigata (wild)	NP by TS + UV irradiated	SW	6% DMSO	5×10^8/ml	Li, 2005
H. rubra (wild)					
H. laevigata (farmed)			6% DMSO + 1% glucose	1.6×10^8/mL	Liu et al., 2014b
H. rubra (farmed)			6% DMSO + 2% glucose		Liu et al.in press b
H. rufescens	NP by 2 M TRIS + 30% hydrogen peroxide		10% GLY		Salinas-Flores et al., 2005
A. purpuratus	SP		10% DMSO + 125mM SUC + 10% yolk	1:3	Espinoza et al., 2010
C. farreri	NP by air exposure + TS		5% DMSO + 5% SUC	1:1	Li et al., 2000
P. yessonesis			16% DMSO	1:3	Yang et al., 2007
M. donacium	pieces of gonads		2M DMSO + 5% SUC + 10% yolk		Dupré and Guerrero, 2011

Table 2.2 Freezing methods and steps, thawing temperatures and outcomes in marine mollusc sperm cryopreservation.

species	freezing method	freezing	thawing temperature	Outcome	references
C. gigas	non-programmable method (NPM)	-15 °C/min from room temperature to -30 °C	70 °C	50% motility; 40% fertilization (10000:1)	Gwo et al., 2003
	PM-one cooling period	at -6 °C/min from 26 °C to -70 °C	74 °C/min up to 26 °C	59% D-larvae (10 - 15:1)	Ieropoli et al., 2004
	dry ice/methanol bath and NPM	(1) chilled to -75 °C with dry ice pellets in methanol for 10 min; (2) 3 cm above LN for 10 min	20 °C	80% fertilization (16000:1)	Adams et al., 2004
	dry ice/methanol bath	chilled to -75°C with dry ice pellets in methanol for 10 min;		90% fertilization (1000 - 10000:1)	Adams et al., 2008
	PM	-16 °C/min to -140 °C (commercial dairy bull sperm freezing method)	40 °C	96% fertilization (2n); 28% fertilization (4n) (4 - 25*10^5:1)	Dong et al., 2005b
	PM-two cooling periods	-5 °C/min to -30 °C; -45 °C/min from -30 °C to -80 °C; 5 min at -80 °C		70% motility; 98% fertilization (10000:1)	Dong et al., 2005c
				17% motility; 7% fertilization (10000:1)	Dong et al., 2006
	PM	commercial dairy bull sperm freezing method		49% fertilization (10000:1)	Dong et al., 2007a
	NPM	3 cm above LN for 20 min	about 60 °C and 20 °C for recovery	92% fertilization (about 250:1); 35% D-larvae	Zhang et al., 2012
C. virginica	PM-one cooling period	-2.5 °C/min from 15 °C to -30 °C; at -30 °C for 5 min	70 °C	22% motility; 57% fertilization (18000:1)	Paniagua-Chávez and Tiersch, 2001
			25 °C	38% intact membranes; 68% functional mitochondria	Paniagua-Chávez et al., 2006
O. edulis		-3 °C /min from 3 °C to -70 °C	55 °C	50% motility	Vitiello et al., 2011

P. fucata martensii	NPM	above LN surface 10 cm or 12 cm to -50 °C	18 ~ 20 °C	32-35% motility[1]	Kawamoto et al., 2007
		above LN surface 10 cm to -50 °C		24% motility	Narita et al., 2008a
				25% motility; 76% fertilization (3200:1)	Narita et al., 2008b
P. marganitifera		above the styrofoam box containing -120 °C LN steam for 10 min	25 °C	86% motility	Lyons et al., 2005
		6 cm above LN for 10-20 min	25 °C	35% motility	Acosta-Salmón et al., 2007
		3 cm above LN for 10 min	27 °C	80% fertilization (100000:1)	Hui et al., 2011
P. martensii		15 cm above LN for 5 min; 5 cm for 5 min	37-40 °C	80% fertilization (40-50:1)	Wang et al., 2006
M. galloprovincialis	PM-three cooling periods	-0.25 °C/min from 20 °C to 2 °C; -1°C/min to -15°C; -6°C /min to -80°C	25 °C	65% motility; 60% fertilization (10:1)	Matteo et al., 2009
P. canaliculus	NPM	3 cm above LN for 10 min	18 °C	80% fertilization (160000:1)	Smith et al., 2012a
H. discus hannai	PM-one cooling period	-50 °C/min from 20 °C to -80 °C	30 °C	65% motility	Kang et al., 2004
H. diversicolor	NPM	-12 or -15 °C/min from 20 °C to -90°C	70 °C	75% motility; 48% fertilization	Gwo et al., 2002
H. laevigata (wild)		at -60 °C for 20 min	50 °C and 18 °C for recovery	94% fertilization (about 3500:1)	Li, 2005
H. laevigata (farmed)		5.2 cm above LN for 10 min	60 °C and 18 °C for recovery	35% motility[1]; 84% fertilization (10000:1)	Liu et al., 2014b
H. rubra (wild)		at -60 °C for 20 min	50 °C and 16 °C for recovery	94% fertilization (about 3500:1)	Li, 2005
H. rubra (farmed)		5.2 cm above LN for 10 min	60 °C and 16 °C for recovery	35% motility[1]; 70% fertilization rate (10000:1)	Liu et al., in press b
H. rufescens		commercial dairy bull sperm	45 °C	29% fertilization (500 -	Salinas-Flores et

		freezing method		5000:1)	al., 2005
A. purpuratus	PM-one cooling period	-8 °C/min from 5 °C to -100 °C	50 °C	45% motility; 58% fertilization (15:1)	Espinoza et al., 2010
C. farreri		-20 °C /min from 0 °C to -80 °C; -80 °C for 2 min;	35 °C	49% motility; 42% fertilization (1000:1)	Li et al., 2000
P. yessonesis	NPM	20 cm above LN for 3 min; 3 cm above LN for 10 min;	24 °C	45% motility; 26% fertilization (500 - 600:1)	Yang et al., 2007
M. donacium	PM-one cooling period	-18 °C/min from 5 °C to -80 °C; 5 min at -80 °C	50 °C for 20s or 30 °C for 40s, then at 20 °C for 4 min	97% fertilization (1000:1)[1]	Dupré and Guerrero, 2011

[1]: Relative motility or fertilization rate, which is calculated by considering the rate in the control as 100%.

2.4 Improvement of marine molluscan sperm cryopreservation techniques

High fresh sperm quality is a prerequisite for cryopreservation as fresh sperm with variable quality could cause unexpected results (Acosta-Salmón et al., 2007). Paniagua-Chávez and Tiersch (2001) has found that although initial sperm motility was similar (> 90%), post-thaw sperm quality differed significantly between individuals in Eastern oysters. Likewise, Dong et al. (2007a) compared the post-thaw sperm fertilization rates in Pacific oysters and found large differences between individuals, ranging from 2 to 90%. Fresh sperm quality may be an important factor influencing cryopreservation success as sperm freezability may be correlated with fresh sperm quality which has been found for some livestock species (Janett et al., 2003; Koonjaenak et al., 2007; Koivisto et al., 2009; Lanes et al., 2010; Coloma et al., 2011; Orgal et al., 2012). Understanding the factors affecting fresh sperm quality, improving the sperm ability to tolerate cryodamage or development of strategies to protect sperm from cryodamage is necessary for the establishment and/or optimization of cryopreservation protocols. Little research in these areas has been performed for marine mollusc and summaries including the findings in livestock and finfish species may pave the way.

2.4.1 Living condition

Broodstock sourced from different living conditions may influence sperm characteristics. In Atlantic cod *Gadus morhua*, spermatocrit, head morphology and seminal plasma differ between wild and farmed stocks, and this is thought to affect the success of sperm cryopreservation (Butts et al., 2010). In Chinook salmon *Oncorhynchus tshawytscha*, Lehnert et al. (2012) demonstrated that the motility,

velocity, longevity and density of sperm derived from farmed stock differed from that derived from wild counterparts. This discrepancy may be due to the aquaculture conditions influence the animals' behaviors or physiology (Skjæraasen et al., 2009). For instance, diets used in aquaculture differ substantially from feed sources in the wild, and diet has a major influence on the development and maturation of the animals. Similar results have been found by our studies of greenlip abalone, showing that when a sperm cryopreservation protocol developed for wild animals was applied to farmed counterparts, low and highly variable post-thaw sperm fertilization rates were achieved. In Australian abalone aquaculture, animals are farmed in land-based shallow concrete tanks, whereas their wild counterparts normally inhabit rock surfaces and steep cliffs at the depths of 2 ~ 40 m. As discussed by Thurston et al. (2002), differences in genetics may also play a role in abalone as loss of genetic variation has been observed in some populations of farmed species, such as *H. iris* (Smith and Conroy, 1992), blacklip abalone and *H. midae* (Evans et al., 2004).

2.4.2 Broodstock nutrition

Sperm quality could be influenced and modulated by broodstock nutrition, and sperm collected from broodstock fed with enriched diets may result in improvement of the sperm ability to tolerate cryodamage (Mocé and Vicente, 2009; Cabrita et al., 2010). In the Senegalese sole *Solea senegalensis*, diets enriched with cholesterol produced a higher ratio of cholesterol to phospholipid in sperm, and the increased ratio was positively correlated with sperm ability to tolerate cold shock (Mocé and Vicente, 2009; Cabrita et al., 2010; Mocé et al., 2010). Furthermore, diets enriched with polyunsaturated fatty acids has improved the sperm characteristics in some finfish species, and may be beneficial for cryoprotection as higher levels of polyunsaturated fatty acids have been detected in cold-shock-resistant species (Cabrita et al., 2010;

Mocé et al., 2010). In marine mollusc, sperm cryopreservation has been investigated in bivalves (oysters, pearl oysters, clams, mussels and scallops) and gastropods (abalone). Bivalves normally feed on particulate organic matter (including phytoplanktons), dissolved amino acids and sugars in seawater (Gosling, 2002). Little information on artificial diets for bivalves has been reported. Caers et al. (2002) has found that feeding the Pacific oysters broodstock with enriched docosahexaenoic acid did not improve the fertilization rate, D-larvae percentage and larval performance. For abalone, farmed abalone broodstock are normally fed with formulated diets while the wild counterparts feed on naturally available seaweeds. Unlike finfish and livestock species, abalone take a long time to feed, increasing the chance of ingredient leaching. Therefore, it would be challenging to enhance the sperm quality by using enriched broodstock diets in marine bivalves and gastropods.

2.4.3 Timing of sperm collection

Time when sperm is collected could influence the sperm quality, including motility, morphology and biochemistry. Changes in these parameters could in turn affect the sperm ability to tolerate cryopreservation (Janett et al., 2003; Lanes et al., 2010). Studies on finfish and livestock species, such as in Brazilian flounder *Paralichthys orbignyanus* (Lanes et al., 2010), *Capra pyrenaica* (Coloma et al., 2011), horse (Janett et al., 2003), bovine (Orgal et al., 2012), buffalo *Bubalus bubalis* (Koonjaenak et al., 2007) and bulls *Bos indicus* and *B. Taurus* (Koivisto et al., 2009), suggest that sperm collected in certain period have higher freezability. It should be noted that in mammals the spermatogenesis is a continuous process. In finfish and mollusc species, on the other hand, the animals experience an annual reproductive cycle involving the growth, ripening of gametes, spawning and redevelopment of gonads. In molluscs, the food availability and temperature are the two key factors

controlling these processes and the stage of spermatogenesis may vary in a natural spawning period (Gosling, 2002). Determination of the timing of sperm collection in a breeding season is of importance as sperm at different stages of spermatogenesis might have a different ability to tolerate cryodamage. In Pacific oysters, for example, Dong et al. (2005b, 2006) suggested that sperm collection during "peak spawning period" could reduce seasonal effects on sperm cryopreservation. In our recent study, farmed greenlip abalone sperm collected at the beginning, middle and end of a natural spawning period have shown different post-thaw sperm fertilization rates (unpublished data).

2.4.4 Age of broodstock and sperm

Influence of broodstock age on sperm motility, concentration and morphology has been found in finfish and livestock species, with older animals having a better ability to tolerate cryopreservation (Lukaszewicz et al., 2003; Lymberopoulos et al., 2010). In marine mollusc, younger animals convert a relatively higher proportion of available energy into somatic growth in comparison with their older counterparts that shift from somatic growth to reproduction (Gosling, 2002). However, no information is available regarding sperm characteristics between broodstock ages. With respect to the age of sperm, the maintenance of sperm quality depends on the sperm collection methods used. With natural spawning, Gwo et al. (2002) has found that sperm fertilization rates decreased significantly from > 90 to 20% after 3 h of natural spawning, accompanied by a greater incidence of embryo deformities in small abalone. Reasons for this phenomenon may be due to the sperm having limited biosynthetic activity and its continued viability greatly depending on catabolic functions, whereas the sperm intrinsic aging process reduces the remaining metabolic activity which leads to sperm quality deterioration (Medeiros et al., 2002). In contrast,

with strip spawning, most sperm are not activated and could be used for days when stored at low temperatures (Dong et al., 2005b). It was also observed by Le Moullac that in blacklip pearl oysters the spawned sperm could enter into a kind of dormancy at low temperature and be stored in a refrigerator for a week with approximately 15% loss in viability when they were reactivated at room temperature and evaluated with the Live/Dead sperm viability kit (Le Moullac, pers. comm.). However, further studies are needed to investigate if they are suitable for cryopreservation.

2.4.5 Sperm quality assessment indicators

Sperm quality assessment indicators, including plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), acrosome integrity (AI) and DNA comet assays have been introduced to reveal the cryopreservation effects on sperm in marine mollusc (Gwo et al., 2003; Salinas-Flores et al., 2005; Paniagua-Chávez et al., 2006; Hui et al., 2011; Liu et al., 2014b). However, knowledge on the structure and biochemical changes of sperm during cryopreservation is limited in marine mollusc, whereas these changes have provided effective information for optimizing the sperm cryopreservation protocols in finfish and livestock species. Differences in membrane biological structure, permeability and fluidity, composition and status of lipids and proteins (Mocé et al., 2010); respiration, mitochondrial ATP and ADP concentrations (Cabrita et al., 2010) and acrosome enzymes leakage (Chauhan et al., 1994) have been used as indicators of quality. An understanding of these characteristics will be important for devising methods to optimize fertilizing ability for marine mollusc. For example, the leakage of lysine from the sperm acrosome is detrimental for fertilization in abalone as this protein is responsible for the dissolution of the egg vitelline layer (Shaw et al., 1993). Previous studies on the ultrastructure of sperm have shown variation in organelles, such as differences in

acrosome and nucleus size between abalone species (Cui et al., 2000; Grubert and Ritar, 2005), and mitochondria numbers between scallops, mussels, abalone, and oysters (Hodgson and Bernard, 1986; Cui et al., 2000; Drozdov et al., 2009). Moreover, a correlation between sperm head morphometric size and freezability has been found in deer (Esteso et al., 2006) and boar (Peña et al., 2005). Therefore, more research on sperm structure and biochemical changes is required to better understand the cryopreservation effects on sperm in marine mollusc.

2.4.6 Sugars, amino acids and vitamins

The addition of sugars, amino acids and vitamins in cryoprotective medium may be an alternative way to improve the sperm ability to tolerate cryodamage as these additives have been found to inhibit lipid peroxidation, resist reactive oxygen species and to reduce osmotic and thermal stress in livestock and finfish species (Li et al., 2003; Khalili et al., 2010; Cabrita et al., 2011; Memon et al., 2013). However, evaluations of these additives in marine mollusc have not been systematically performed, although some have been selected as co-CPAs. In our recent study, the addition of glucose has significantly improved the post-thaw sperm PMI and MMP in farmed greenlip abalone (Liu et al., 2014b) and replacement of glucose with galactose or fructose has shown a similar cryoprotective effect (Liu et al., 2014a). Moreover, the addition of L-ascorbic acid or glycine could significantly enhance post-thaw sperm PMI and AI in farmed greenlip abalone (Liu et al., 2014a), with higher values being achieved with glycine treatments. This may be because glycine in the medium could (1) form a layer on the sperm surface which could reduce the amount of permeable CPA entering into sperm, and minimize the osmotic or toxic stress (Li et al., 2003; Khalili et al., 2010) and (2) stabilize sperm membrane and prevent lipid peroxidation (Khalili et al., 2010).

2.5 Conclusion

Sperm cryopreservation is a valuable technique that has been widely applied and commercialized in livestock species. In contrast, this technique is relatively new in marine mollusc. Although protocols have been developed for a few economically important molluscan species, they have not been routinely used by the industry. Economic considerations also influence the application of sperm cryopreservation techniques in marine mollusc, as most aquaculture enterprises are relatively small, and farm- or regional-based genetic improvement programs have only recently been initiated.

The published results clearly indicate that salt balanced solution is a suitable extender for species their sperm can be collected by strip spawning, such as oysters and pearl oysters. However, seawater should be the choice for those species their sperm are normally collected using natural spawning such as mussels, abalone and scallops. DMSO is a common CPA suitable for sperm cryopreservation in most molluscan species. In comparison, non-programmable freezing technique normally produces better results than programmable freezing technique in marine molluscan sperm cryopreservation studies.

Reliability is a practical issue that needs to be addressed as research on sperm cryopreservation in marine mollusc has mainly focused on the optimization of cryopreservation protocols while less on the factors affecting fresh sperm qualities. Knowledge from livestock and finfish species has suggested that physiological and environmental factors influence the quality of sperm collected, thus the reliability of sperm cryopreservation. In addition, a better understanding of sperm structure and biochemical composition changes could provide necessary knowledge for assistance

in the improvement of post-thaw sperm quality. It is expected that sperm cryopreservation techniques in marine mollusc could be greatly improved and better applications would then be found by aquaculture businesses.

CHAPTER 3 : CRYOPRESERVATION OF SPERM IN FARMED AUSTRALIAN GREENLIP ABALONE *HALIOTIS LAEVIGATA*[*]

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3.1 Abstract

This study investigated factors important to the development of the liquid nitrogen (LN) vapour sperm cryopreservation technique in farmed greenlip abalone *Haliotis laevigata*, including (1) cryoprotectant agent (CPA) toxicity; (2) cooling temperature (height above LN surface); (3) thawing temperature; (4) sperm to egg ratio; and (5) sugar supplementation, using sperm motility, fertilization rate or integrity/potential of sperm components and organelles as quality assessment indicators. Results suggested that among the single CPAs evaluated 6% dimethyl sulfoxide (DMSO) would be the most suitable for sperm cryopreservation in this species. The highest post-thaw sperm motility was achieved with the sperm that had been exposed to LN vapour for 10 min at 5.2 cm above the LN surface, thawed and recovered in 60 ºC and 18 ºC seawater bathes respectively after at least 2 h storage in LN. The highest fertilization rates were achieved at a sperm to egg ratio of 10000:1 or 15000:1. Addition of 1% glucose or 2% sucrose produced significantly higher post-thaw sperm motility than 6% DMSO alone. Among the three cryoprotectant solutions further trialled, 6% DMSO + 1% glucose produced the highest fertilization rate of 83.6 ± 3.7%. Evaluation of sperm has shown that the addition of glucose could significantly improve the sperm plasma membrane integrity and mitochondrial membrane potential. These results demonstrated a positive role of glucose in the

improvement of sperm cryopreservation in farmed greenlip abalone.

Keywords: farmed greenlip abalone, *Haliotis laevigata*, sperm cryopreservation, glucose

3.2 Introduction

The abalone aquaculture industry in Australia has developed rapidly in the last decade with production reaching about 681 metric tons worth about 23 million dollars in the financial year 2010-2011 (Australian fisheries statistics, 2012). Greenlip abalone *Haliotis laevigata* is the most valuable species in this sector, accounting for 70% of the total abalone production (Kube et al., 2007), with farms having been established in South Australia, Victoria, Tasmania and Western Australia. Research to improve abalone production has focused on system design (Freeman, 2001), optimization of diet (Coote et al., 1996) and environmental parameters (Harris et al., 1997, 1998). The opportunity now exists to make use of genetic improvement techniques to further advance the abalone aquaculture industry, and selective breeding programs have been initiated in this species (Kube et al., 2007; Li, 2008; Robinson et al., 2010). However, due to restrictions on translocation of live abalone between states in Australia, each state needs to establish enough families to allow for the long-term maintenance of genetic diversity, avoid inbreeding and meet the demand on new traits in the future. The efficiency of these programs is also reduced by the following biological and technical limitations: (1) difficulty in achieving the desired mating as selected males and females cannot be induced to spawn synchronously; (2) short spawning window period in a breeding season; and (3) risks associated with keeping superior broodstock alive and healthy. These limitations are not unique to the abalone aquaculture industry, because they have been experienced in other species and mitigated by the development and application of sperm cryopreservation techniques (Purdy, 2006; Mocé and Vicente, 2009; Cabrita et al., 2010).

Sperm cryopreservation has been extensively used in livestock animal breeding programs and for distributing genetically improved sperm for commercial production (Purdy, 2006; Mocé and Vicente, 2009), and has become a near-billion-dollar business world-wide (Tiersch, 2008). In aquatic species, sperm cryopreservation research has mainly been conducted in fish species (Suquet, 2000; Muchlisin, 2005; Cabrita et al., 2010). Studies on marine mollusc have increased substantially over the last decade, especially in oysters (Adams et al., 2004, 2008; Dong et al., 2005b, c, 2006, 2007a, b). In abalone, this technique has been investigated in small abalone *H. diversicolor* (Tsai and Chao, 1994; Gwo et al., 2002), disc abalone *H. discus hannai* (Kang et al., 2004), red abalone *H. rufescens* (Salinas-Flores et al., 2005), wild greenlip abalone and wild blacklip abalone *H. rubra* (Li, 2005), resulting in ~90% fertilization rate in both small and wild greenlip abalone (Tsai and Chao, 1994; Zhu et al., 2014). In the study on wild greenlip abalone, two cryoprotectant agents (dimethyl sulfoxide and glycerol) were evaluated and a cryopreservation protocol was developed (Zhu et al., 2014). However, when this protocol was applied to farmed stocks, low and highly variable fertilization rates were experienced, hindering the adoption of this technique (Li, pers. comm.).

It has been shown in the published data that sperm quality, such as motility, viability and the integrity or potential of components and organelles, could be compromised by the cryopreservation processes, thus reducing sperm competency (Adams et al., 2003; Salinas-Flores et al., 2005; Paniagua-Chávez et al., 2006; Smith et al., 2012b; Zhang et al., 2012). However, this reduction in sperm quality could be improved by the addition of cryoprotectant agents (CPAs). In some species, this could be further improved if permeable CPAs (e.g. dimethyl sulfoxide and propylene glycol) are used in combination with non-permeable CPAs (e.g. sugars) (Purdy, 2006; Si et al., 2006;

Naing et al., 2010; Jafaroghli et al., 2011). For example, the post-thaw sperm motility, viability and membrane integrity were significantly improved by the addition of trehalose in rams (Jafaroghli et al., 2011) and in some marine molluscan species such as blacklip pearl oysters *Pinctada marganitifera* (Lyons et al., 2005). Furthermore, disaccharides gave better protection than monosaccharides in boars (Gómez-Fernández et al., 2012). The aim of this study was to establish a sperm cryopreservation protocol for farmed greenlip abalone. We have evaluated most permeable CPAs and key factors affecting sperm cryopreservation in molluscan species, and assessed if the addition of sugars could further improve the post-thaw sperm quality.

3.3 Materials and methods

3.3.1 Broodstock

Farmed mature greenlip abalone were supplied by SAM Abalone in Port Lincoln, South Australia and transported in a foam box by air to the Aquatic Sciences Centre, South Australian Research and Development Institute (SARDI), Adelaide, South Australia. The animals were about 3 years old and 122.0 ± 8.9 g in total body weight. Upon arrival they were cleaned with 5 μm filtered seawater (FS) and their gender and gonad condition checked. The colour of the male gonad is milky white while that of the female is dark green. Animals with a large and swollen gonad were selected to acclimatize in tanks for a week prior to spawning (Heasman and Savva, 2007). The tanks were on a flow through system with 5 μm FS at 15.0 ~16.0 ºC. Over this period, the abalone were fed twice a week with the artificial diet provided by EP Aquafeeds (Adelaide, South Australia) and the system was cleaned when needed.

3.3.2 Gamete collection

Five to ten male (and female if needed) abalone were used in each experiment. The abalone were induced to spawn by raising the seawater temperature by 2 ~ 3 ºC for about 2 h before the ultraviolet (UV) light connected to the water supply system was turned on. Immediately upon the commencement of spawning, the water supply to the tank was changed from UV irradiated seawater to 5 μm FS of the same temperature. To collect concentrated sperm, the water supply to the tank was turned off approximately 5 to 10 min after the commencement of spawning. The tank was then emptied and the abalone were left undisturbed in the tank for sperm collection. If abalone were dislodged from the tank wall, they were dried with paper towel and put on a rack with their shell facing down. Sperm were then collected by placing a shallow container underneath. Sperm collected from abalone that spawned earlier were stored on ice until sperm from at least 3 individuals were collected. An equal volume of sperm from each male was then pooled. Three sperm pools were established using different males respectively. The sperm concentration was determined using 3 subsamples per pool. Each subsample was diluted 100 times and counted under a light microscope with a haemocytometer. The sperm concentration was then standardized to 1.6×10^8 mL^{-1}. Sperm samples with initial motility above 80% were used in the subsequent experiments. The time interval from when the sperm from the first male was collected to when the pooled sperm was used in the subsequent experiments was kept as short as possible and was never longer than 2 h.

Eggs in spawning tanks were gently poured into a sieve set consisting of a 300 μm upper sieve to remove large debris and a 90 μm lower sieve partly immersed in 5 μm FS to retain the eggs. The eggs were gently rinsed and then washed into a settlement beaker. After 15 min the eggs on the bottom were transferred into another container

and mixed gently by hand. One millilitre egg suspension was taken and diluted 100 times before a 1 mL subsample was used to account the egg density under a binocular microscope. This estimation was repeated three times per collection. The eggs used for the fertilization rate evaluation were never older than 2 h post spawning.

3.3.3 Chemical solution preparation

3.3.3.1 Cryoprotectant agent (CPA)

Dimethyl sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG), glycerol, sucrose, glucose and trehalose used in this study were AR grade and purchased from Sigma-Aldrich Pty Ltd. Stock solutions were prepared in 5 μm FS at a concentration twice that of the final concentration required. When the stock solution was mixed with sperm at a 1:1 ratio in the experiments, the required final concentration was produced.

3.3.3.2 Fluorescent agent

A LIVE/DEAD sperm viability kit (L-7011) for plasma membrane integrity (PMI) evaluation and LysoTracker green DND-26 (LYSO-G) kit (L-7526) for acrosome integrity (AI) evaluation were purchased from Invitrogen Australia. Rhodamine 123 (Rh 123) for mitochondrial membrane potential (MMP) evaluation and propidium iodide (PI) used for AI and MMP evaluation were purchased from Sigma-Aldrich Pty Ltd. The working solution of 2 μM SYBR14 and 200 μM PI in the LIVE/DEAD sperm viability kit was prepared by diluting with 5 μm FS. LYSO-G in LysoTracker green DND-26 kit is 1000 μM and used directly. Rh123 was prepared in 5 μm FS at a concentration of 10 μM. PI was prepared in 5 μm FS at a concentration of 3000 μM or 130 μM for AI and MMP evaluations, respectively (Thomas et al., 1997; He and

Woods, 2004; Salinas-Flores et al., 2005; Smith et al., 2012b).

3.3.4 Equipment setup

In this study, a styrofoam box (39.0 cm x 24.5 cm x 35.5 cm), a foam rack with different heights (1.3, 2.6, 3.9, 5.2 and 6.5 cm) and a digital thermometer (Thermo Scan, Eutech Instruments, Singapore) with a low temperature probe were used. Liquid nitrogen (LN) was added into the styrofoam box with a LN withdrawal device to a depth of about 2 cm. The rack was then placed into the styrofoam box, floating on the LN surface. After 0.25 mL straws (Minitube, Germany) containing the sperm + cryoprotectant solution mixture were placed on the rack the lid of styrofoam box was closed partially to maintain the constant flow of vaporized LN during freezing (Farstad, 2010; Ping et al., 2011). The temperatures at different heights were measured by the digital thermometer.

Two seawater baths were used in the experiments to thaw and recover sperm at different temperatures. The required temperature (40 ~ 80 °C) in the thawing bath was achieved by mixing ambient and boiled seawater. The 18 ºC temperature required in the recovery bath was achieved by mixing ambient and cold seawater.

3.3.5 Sperm quality evaluation methods

The resultant sperm quality was assessed by measuring motility, fertilization rate or integrity/potential of sperm components or organelles.

The motility was determined by diluting the sperm suspension 5 times with 5 µm FS and then counting the number of active sperm out of 100. Sperm moving forward progressively were counted as active sperm while those vibrating or not moving at all were counted as dead sperm (Gwo et al., 2002). Each sample was assessed by two independent observers. To standardize across the experiments, the percentage of

fresh sperm motility was set to 100% and the post-thaw sperm motility value was calculated as a percentage of the fresh sperm motility (Kawamoto et al., 2007).

During fertilization, the required volume of concentrated eggs was taken using a pipette. They were then fertilized by the thawed sperm and stirred by hand gently. Ten minutes later the eggs were washed gently on a 90 µm sieve. The eggs were then reared in a 500 mL container. The fertilization rate was determined microscopically 4 h post-fertilization by counting the number of fertilized eggs out of a sample of 100. Eggs with two or multiple cells were counted as fertilized eggs while the rest was counted as unfertilized or dead eggs.

Sperm PMI, MMP and AI were evaluated by SYBR14/PI, Rh123/PI and LYSO-G/PI methods respectively (Thomas et al., 1997; He and Woods, 2004; Salinas-Flores et al., 2005; Smith et al., 2012b). The thawed sperm were diluted to 1 x 10^7 sperm/mL and 1 mL diluted sperm were then stained. For PMI and MMP, 100 µL SYBR14 and Rh123 were added for 20 min, respectively, and then 100 µL PI for further 10 min. For AI, 5 µL LYSO-G was added for 30 min, and then 9 µL PI for further10 min. All staining was carried out at the room temperature. The samples were observed under an Olympus BX60 fluorescence microscope. Organelles of viable sperm emit green fluorescence, while dead sperm emit red fluorescence. The integrity or potential of an organelle was expressed as the percentage of viable sperm.

3.3.6 Experiments

In this study, all the experiments were repeated three times using sperm from different pools, while the repeat was further replicated three times using sperm from the same pool.

3.3.6.1 Effects of CPA types and concentrations on sperm motility

In this experiment, four CPAs were evaluated at five final concentrations. Pre-cold fresh sperm (on ice) were mixed with pre-cold DMSO, EG, PG or glycerol (on ice) at a final concentration of 4, 8, 12, 16 or 20% and maintained on ice. After 10 min equilibration, sperm subsamples were collected to assess motility.

3.3.6.2 Effects of equilibration times on sperm motility

EG and PG at a final concentration of 8% and DMSO at 8 and 12% were selected according to the results from the previous experiment and evaluated with four equilibration times (5, 10, 30 and 60 min). Two equilibration times (10 and 20 min) were further assessed using 8% EG, PG and DMSO. The treatments were conducted on ice and evaluated in the same way as in section *3.3.6.1*.

3.3.6.3 Effects of rack heights on sperm motility

An equilibration time of 10 min and 8% DMSO, EG and PG were selected in this experiment. Five heights (1.3, 2.6, 3.9, 5.2 and 6.5 cm) above the LN surface were evaluated in the styrofoam box. After equilibration on ice, the sperm and cryoprotectant solution mixtures were transferred into 0.25 mL straws and put on racks at different heights. The straws were exposed to LN vapour for 10 min before being put into LN for at least 2 h storage. The sperm were thawed and recovered in 50 °C and 18 °C seawater baths, respectively. Subsamples from post-thaw straws were collected to assess sperm motility.

3.3.6.4 Effects of thawing temperatures on sperm motility

In the previous experiment, sperm frozen at 5.2 cm height resulted in the highest post-thaw sperm motility. Thus this height was used in this and subsequent experiments. In this experiment, five thawing temperatures (40, 50, 60, 70 and 80 °C)

with three DMSO final concentrations (6, 8 and 10%) were evaluated. The other procedures were the same as described in section *3.3.6.3*.

3.3.6.5 Comparison of sperm to egg ratios on fertilization rate

The treatments producing the highest post-thaw sperm motility (6% DMSO, 5.2 cm rack height and 60 °C thawing temperature) in the previous experiments were selected in this experiment to test the effect of sperm to egg ratios on fertilization rate. Six ratios (10:1, 100:1, 1000:1, 10000:1, 15000:1 and 20000:1) were evaluated. The other procedures were the same as in section *3.3.6.4* except that the sperm quality was evaluated using fertilization rates as described in section *3.3.5*.

3.3.6.6 Effects of sugar types and concentrations on sperm motility and fertilization rate

In this experiment, the effects of adding sugars (sucrose, glucose and trehalose) into 6% DMSO were evaluated at five concentrations (1, 2, 3, 4 and 5%). The parameters optimized in previous experiments were also applied (5.2 cm rack height and 60 °C thawing temperature). The effects of different sugar types and concentrations were assessed using post-thaw sperm motility. Further evaluation of fertilization rate was conducted using 6% DMSO and its combination with 1% glucose or 2% sucrose at a sperm to egg ratio of 10000:1. The other procedures were the same as described in section *3.3.6.5*.

3.3.6.7 Comparison of PMI, MMP and AI between sperm cryopreserved with 6% DMSO and 6% DMSO + 1% glucose

In this experiment, PMI, MMP and AI were compared between sperm cryopreserved with 6% DMSO and 6% DMSO + 1% glucose to assess the protection effects of glucose on sperm components or organelles. The other procedures were the same as

described in section *3.3.6.6*. Procedures for evaluating the integrity/potential of sperm components or organelles were described in section *3.3.5*.

3.3.7 Statistical analysis

The results in this study were presented as mean ± standard deviation (SD). However, they were arcsine or log transformed for statistical analyses using SPSS 20. A t test for independent samples was applied to analyse the impacts of the addition of 1% glucose in *section 3.3.6.7*. Two-way analysis of variance (ANOVA) was applied to analyse the results in the experiments on CPA type and concentration, equilibration time, rack height, thawing temperature and sugar type and concentration on sperm motility. One-way ANOVA was applied in the other experiments. The Least-Significant Difference (LSD) comparison test was used when significance was observed. Differences were considered statistically significant at $P < 0.05$.

3.4 Results

3.4.1 Effects of CPA types and concentrations on sperm motility

Results from this experiment showed that farmed greenlip abalone sperm were sensitive to glycerol as all concentrations evaluated in this study resulted in very low or no motility (Table 3.1). When the DMSO concentration was higher than 12% and EG and PG were higher than 8%, motilities of less than 35% were produced.

Table 3.1 Sperm motilities (%) after exposure to CPAs at different concentrations for 10 min on ice, $n = 3$.

CPA	Final concentration (%)				
	4	8	12	16	20
DMSO	81.1±1.4[a]	73.6±2.7[b]	56.9±3.1[c]	22.1±3.5[d]	12.1±1.5[e]
EG	71.3±1.3[a]	42.6±4.9[b]	25.2±3.6[c]	18.6±3.6[d]	5.9±3.3[e]
PG	68.8±1.2[a]	47.1±1.3[b]	34.6±1.4[c]	15.2±2.4[d]	7.3±3.4[e]
Glycerol	1.6±0.6[a]	1.6±0.9[a]			

Different letters within each CPA indicate significant difference between different concentrations.

3.4.2 Effects of equilibration times on sperm motility

When equilibration time was extended to 30 min, all CPAs resulted in less than 20% sperm motility while 10min exposure produced a similar level of sperm motility as those reported in section *3.3.1* (Fig. 3.1). Further investigation with equilibration times of 10 and 20 min showed no significant difference among 8% EG, PG and DMSO and all treatments achieved higher than 55% sperm motility (Fig. 3.2). In 8% PG and DMSO treatments, motilities were significantly higher in sperm equilibrated for 10 min than those equilibrated for 20 min.

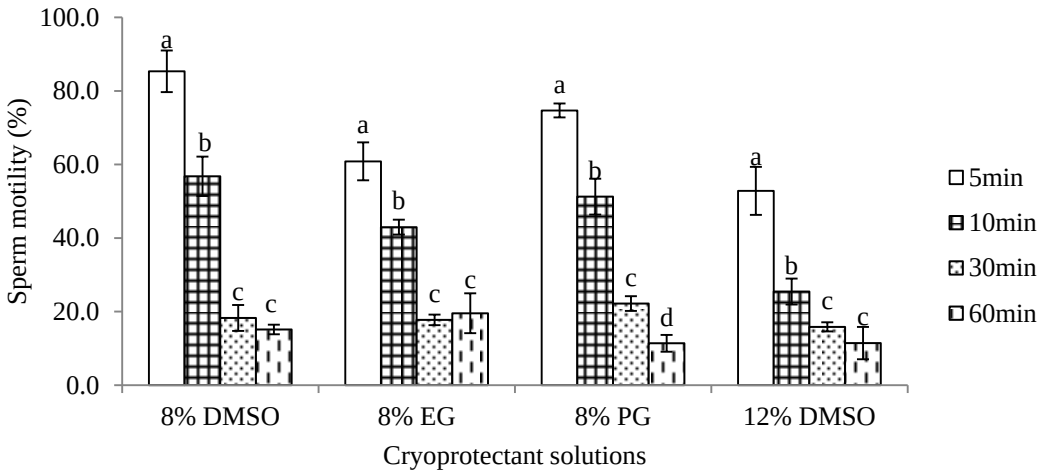

Figure 3-1 Sperm motilities (%) after exposure to selected cryoprotectant solutions at equilibration time of 5, 10, 30 and 60 min, *n* = 3. Different letters within each cryoprotectant solution indicate significant difference between different equilibration times.

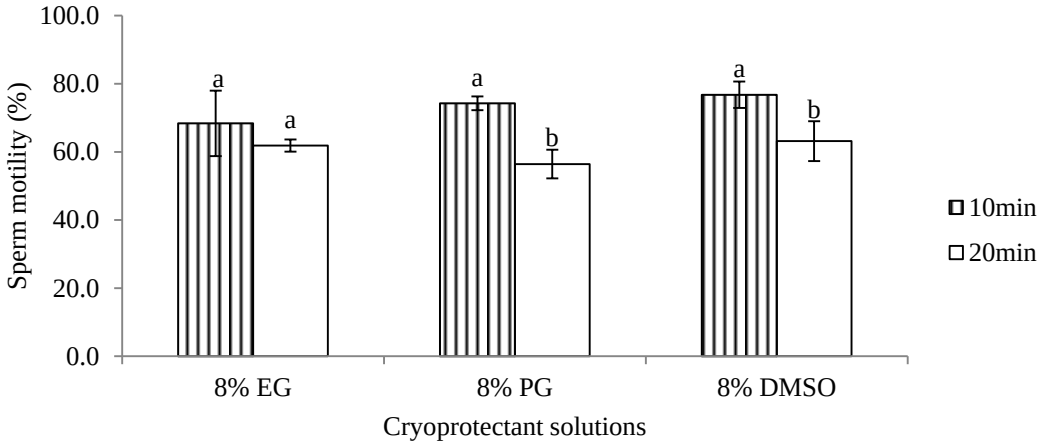

Figure 3-2 Sperm motilities (%) after exposure to 8% EG, PG or DMSO at equilibration time of 10 and 20 min, *n* = 3. Different letters within each cryoprotectant solution indicate significant difference between equilibration times.

3.4.3 Effects of different rack heights on sperm motility

Eight percent DMSO produced a higher post-thaw sperm motility than EG and PG of the same concentration at each height evaluated (Fig. 3.3). Sperm motility was the greatest at 5.2 cm rack height, although it did not differ significantly from 1.3 and 2.6 cm rack height treatments.

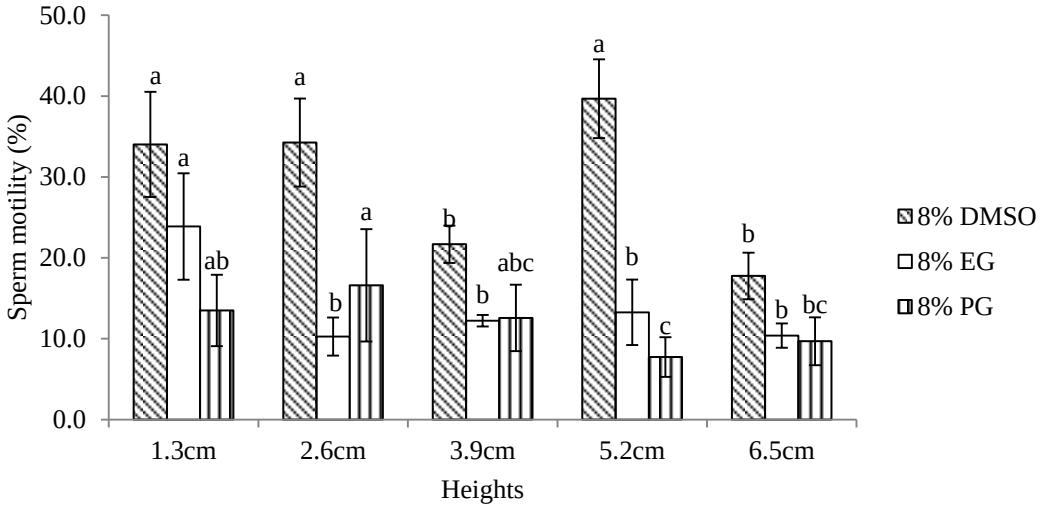

Figure 3-3 Post-thaw sperm motilities (%) after being frozen at different rack heights in 8% DMSO, EG or PG for 10 min and stored in LN for at least 2 h, n = 3. Different letters within each cryoprotectant solution indicate significant difference between different rack heights.

3.4.4 Effects of thawing temperatures on sperm motility

The post-thaw sperm motility was affected by both DMSO concentrations and thawing temperatures (Fig. 3.4). Six percent DMSO produced a higher post-thaw sperm motility than 8 and 10% DMSO treatments at each thawing temperature evaluated. The highest post-thaw sperm motility of 41.8 ± 2.4% was achieved with cryopreservation in 6% DMSO and thawing at 60 °C, which was significantly higher than at other temperatures.

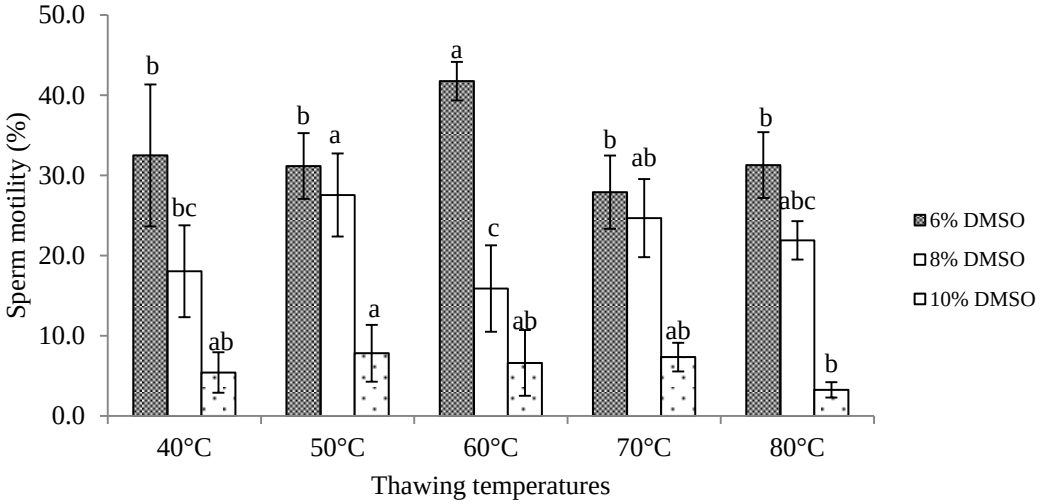

Figure 3-4 Post-thaw sperm motilities (%) after cryopreservation in 6, 8 or 10% DMSO and thawed at different temperatures, *n* = 3. Different letters within each cryoprotectant solution indicate significant difference between different thawing temperatures.

3.4.5 Comparison of sperm to egg ratios on fertilization rate

The fertilization rate increased with the increase in sperm to egg ratios, resulting in the highest fertilization rate of 63.0 ± 10.6% at the ratio of 15000:1 (Fig. 3.5). However, this rate did not significantly differ from those achieved with sperm to egg ratio of 10000:1 (59.1 ± 15.9%), although it was significantly higher than at other sperm to egg ratios (less than 40%) (Fig. 3.5).

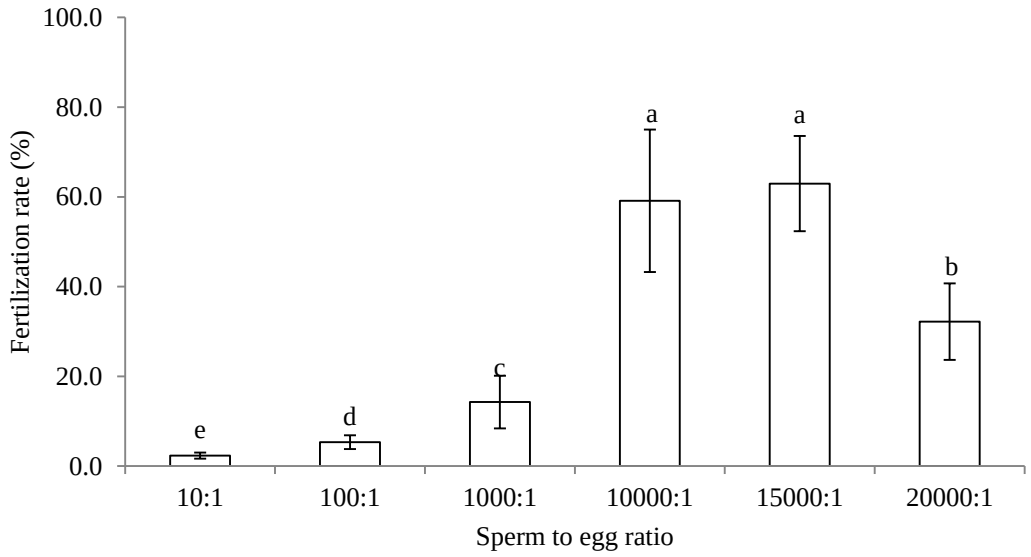

Figure 3-5 Post-thaw sperm fertilization rates (%) at different sperm to egg ratios, *n* = 3. Different letters indicate significant difference.

3.4.6 Effects of sugar types and concentrations on sperm motility and fertilization rate

The post-thaw sperm motilities were compared to evaluate the effects of sugar additions (Fig. 3.6). No significant difference was found in the post-thaw sperm motility among the three controls (6% DMSO). The addition of 1% glucose or 2% sucrose improved the post-thaw sperm motility significantly ($P < 0.001$ and 0.05, respectively), while no improvement was revealed in the addition of trehalose (Fig. 3.6). Further investigation of selected cryoprotectant solutions (6% DMSO, 6% DMSO + 1% glucose and 6% DMSO + 2% sucrose) showed that 6% DMSO + 1% glucose produced the highest fertilization rate of 83.6 ± 3.7% (Fig. 3.7).

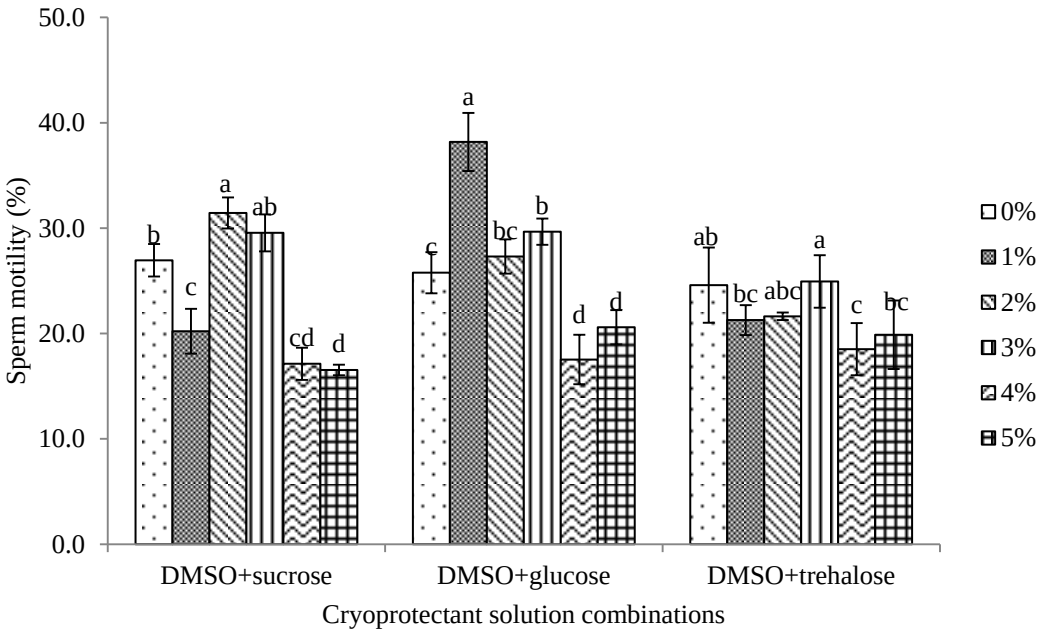

Figure 3-6 Post-thaw sperm motilities (%) after addition of different types and concentrations of sugar into 6% DMSO, *n* = 3. Different letters within each CPA combination indicate significant difference between different sugar concentrations.

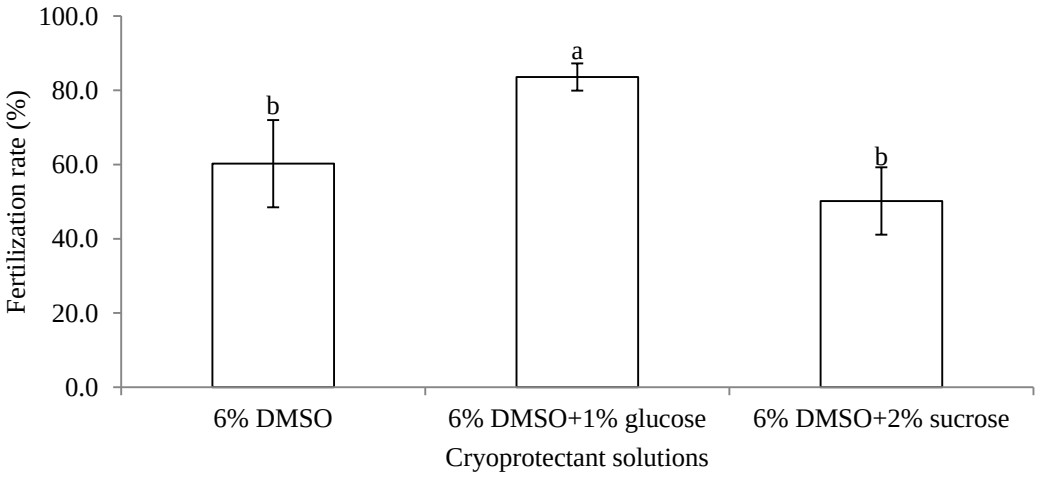

Figure 3-7 Comparison of post-thaw sperm fertilization rates in 6% DMSO and its combination with 1% glucose or 2% sucrose, *n* = 3. Different letters indicate significant difference.

3.4.7 Comparison of PMI, MMP and AI between sperm cryopreserved with 6% DMSO and 6% DMSO + 1% glucose

The PMI and MMP were significant higher in sperm cryopreserved in 6% DMSO + 1% glucose than in 6% DMSO alone (Table 3.2). However, no significant difference was found in AI between these two treatments.

Table 3.2 Comparison of plasma membrane integrity (PMI), mitochondrial membrane potential (MMP) and acrosome integrity (AI) between sperm cryopreserved in 6% DMSO and 6% DMSO + 1% glucose, $n = 3$.

Cryoprotectant solution	Sperm component and organelle		
	PMI	MMP	AI
6% DMSO	49.4 ± 1.6%[a]	18.3 ± 1.9%[a]	43.4 ± 10.0%[a]
6% DMSO + 1% glucose	55.8 ± 1.5%[b]	24.9 ± 0.9%[b]	49.9 ± 4.7%[a]

Different letters within each parameter evaluated indicate significant difference between these two cryoprotectant solutions.

3.5 Discussion

This study has optimized key parameters affecting the sperm cryopreservation in farmed greenlip abalone and achieved 60% fertilization rate using 6% DMSO. This rate was further improved to higher than 80% by addition of 1% glucose, a rate which would be suitable for commercial abalone production and selective breeding. Evaluation of sperm plasma membrane integrity and mitochondria membrane potential suggested that glucose plays a positive role in maintaining sperm quality during the cryopreservation in this species.

Low toxicity is one of the essential characters of an effective CPA (Ieropoli et al., 2004; Lyons et al., 2005; Dong et al., 2007a). Of the CPAs evaluated in this study, sperm of farmed greenlip abalone were extremely sensitive to glycerol, even at the lowest concentration of 4%. This was consistent with the results in small abalone where glycerol reduced sperm motility to < 25% at the concentration of 5% (Gwo et al., 2002). Similar results have also been found in other species, such as Pacific oysters *Crassostrea gigas*, Japanese pearl oysters and European flat oysters *Ostrea edulis* (Ieropoli et al., 2004; Dong et al., 2007b; Kawamoto et al., 2007; Vitiello et al., 2011). On the other hand, glycerol has been shown to be suitable for sperm cryopreservation in Arctic charr *Salvelinus aplinus*, filefish *Thamnaconus septentrionalis*, giant abalone *H. gigantean*, disc abalone and red abalone (Matsunaga et al., 1983; Piironen, 1993; Kang et al., 2004; Salinas-Flores et al., 2005), suggesting that the toxicity of glycerol might be species specific. In this study, farmed greenlip abalone sperm were also sensitive to other CPAs higher than 12%, which agreed with the findings in most marine molluscan species (Gwo et al., 2002; Adams et al., 2004; Salinas-Flores et al., 2005; Acosta-Salmón et al., 2007; Dong et

al., 2007a, Kawamoto et al., 2007; Matteo et al., 2009; Smith et al., 2012a; Zhu et al., 2014).

The equilibration time should be sufficient for CPA to enter into sperm, but not so much so that toxic effects on the sperm are expressed during cryopreservation (Sansone et al., 2005; Matteo et al., 2009). In the current study, sperm motility decreased significantly after 30 min equilibration in all 8% CPAs evaluated and was lowered further with the increase in CPA concentrations. A similar trend was also found in small abalone (Gwo et al., 2002). After 20 min equilibration in this study, 60% sperm motility was retained, which agreed with the findings in other marine mollusc where equilibration times between 10 and 20 min were normally selected (Ieropoli et al., 2004; Dong et al., 2005b; Salinas-Flores et al., 2005; Paniagua-Chávez et al., 2006; Acosta-Salmón et al., 2007).

At the same CPA concentrations, DMSO showed higher cryoprotective effects than other CPAs (Fig. 3.3). Suquet et al. (2000) and Sansone et al. (2005) have suggested that this is because the permeability of DMSO is less sensitive to low temperatures and can enter sperm and interact with membrane phospholipids quickly, thus providing protection during freezing (Suquet et al., 2000; Sansone et al., 2005). Further, of the DMSO concentrations compared, 6% resulted in the highest post-thaw motility, agreeing with the level reported by Zhu et al. (2014) in wild greenlip abalone.

Most cryoinjuries caused by freezing occur between 0 and -40 °C (Chao and Liao, 2001; Kawamoto et al., 2007). As intracellular ice formation and solute concentration increase are the major factors contributing to sperm damage over this freezing temperature range, an ideal cooling rate should be neither too fast, nor too slow. Too fast could induce the intracellular ice formation, while too slow could

extend sperm's exposure to the increased solute concentrations (Gao and Critser, 2000; Chao and Liao, 2001; Ieropoli et al., 2004; Kawamoto et al., 2007). In this study, 5.2 cm above the LN surface resulted in the highest post-thaw sperm motility. The temperature measured after 10min exposure to LN vapour at this height was -103 °C. This height was lower than those found in Japanese pearl oysters, scallops (*Patinopecten yessoensis* and *Chlamys farreri*) and blacklip pearl oysters (Yang et al., 1999; Acosta-Salmón et al., 2007; Kawamoto et al., 2007), but higher than in Greenshell mussels *Perna canaliculus* (Smith et al., 2012a).

Thawing temperature is another important factor affecting the success of sperm cryopreservation because high thawing temperatures could inhibit the re-crystalization of the internal ice, while low thawing temperatures might prevent osmotic stress on sperm due to extracellular solution fast melting caused by high thawing temperatures (Gao and Critser, 2000; Herráez et al., 2012). Thawing temperatures between 55 and 60 °C have produced acceptable results (>80% fertilization rate) in Pacific and American *C. virginica* oysters (Zell et al., 1979; Yankson and Moyse, 1991). Among the thawing temperatures evaluated in this study, 60 °C also produced the highest post-thaw sperm motility. However, this differed from the results in wild greenlip abalone where the fertilization rates of sperm thawed at 50 °C were significantly higher than those at 60 °C (Zhu et al., 2014). Reasons for this discrepancy between wild and farmed stocks are unknown. Further experiments would be needed to directly compare the optimal thawing temperature between them. In this study, thawing temperatures of 40 and 70 °C were suboptimal, whereas they were optimal in pearl oysters *Pinctada martensii* and small abalone, respectively (Gwo et al., 2002; Wang et al., 2006).

In comparison with freshly collected sperm, a higher sperm to egg ratio is normally

needed in fertilization to compensate for cryodamage of the sperm, such as the impairment of plasma membrane, mitochondria and/or acrosome (Salinas-Flores et al., 2005; Paniagua-Chávez et al., 2006; Zhang et al., 2012). When fresh sperm were used in greenlip abalone >80% fertilization rates could be produced at a sperm to egg ratio of ≥ 100:1 (Babcock and Keesing, 1999). In this study, sperm to egg ratios less than 1000:1 resulted in low post-thaw sperm fertilization rates. Ratios of this order were not considered suitable for fertilization after cryopreservation in other marine molluscan species (Gwo et al., 2003; Adams et al., 2008; Narita et al., 2008b; Hui et al., 2011; Smith et al., 2012a). In this study, the highest fertilization rate was achieved at a ratio of 10000:1 or 15000:1. A similar ratio also resulted in the highest fertilization rate in Pacific oysters (Gwo et al., 2003; Adams et al., 2008). However, they vary between species or studies. For example, sperm to egg ratios lower than these have achieved the highest fertilization rates in wild greenlip abalone (Zhu et al., 2014) and small abalone (Tsai and Chao, 1994). Ratios higher than 15000:1 normally resulted in lower fertilization rates in many species, such as Pacific oysters (Gwo et al., 2003; Adams et al., 2004, 2008) and Japanese pearl oysters (Narita et al., 2008b). In this study, a ratio of 20000:1 produced about half the fertilization rate of the 10000:1.

Sperm collected from farmed and wild animals have found some different characters and these differences might result in different freezability. In cod *Gadus morhua*, for example, Skjæraasen et al. (2009) have found that the mean curvilinear velocity, percent motile sperm and percent progressive sperm in wild broodstock were superior to farmed counterparts (Skjæraasen et al., 2009). These characters have shown positive correlations with fertilization rates in other studies (Tsai and Chao, 1994; Chao and Liao, 2001; Matteo et al., 2009). Skjæraasen et al. (2009) further

speculated that aquaculture conditions could change the animals' behaviour, leading to a dominance hierarchy modification in cod, which may explain the reason why sperm quality collected in farmed stocks was inferior to wild stocks (Skjæraasen et al., 2009). Moreover, nutritional deficiency may be another factor contributing to sperm quality differences between wild and farmed stocks as wild broodstock are normally considered to have balanced diet, leading to the high sperm quality (Meunpol et al., 2005; Skjæraasen et al., 2009). Male age also has been found to influence the fresh sperm quality in some species, which may further affect the sperm freezability (Ceballos-Vázquez et al., 2003; Long et al., 2010; Tsakmakidis et al., 2012). In this study, 6% DMSO produced the highest fertilization rate of 60% among the single CPAs evaluated. This DMSO concentration also resulted in the best fertilization rate of >90% in wild greenlip abalone in the study by Zhu et al. (2014). The reasons causing this 30% difference in fertilization rate between wild and farmed greenlip abalone were unclear, although might be due to the factors discussed above. The wild greenlip abalone used in Li's study were at least 7 years old. Wild abalone of this age normally inhabit rock surfaces and steep cliffs in depths between 2 and 40 meters and feed on naturally available seaweeds (Freeman, 2001). The farmed counterparts used in this study, on the other hand, were 3 years old and were cultured in land-based shallow concrete slab tanks and fed artificial diets (Marshall, per. comm.).

The addition of sugars into CPA is a potential strategy to improve sperm quality during cryopreservation and has been applied in some species (Lyons et al., 2005; Si et al., 2006; Naing et al., 2010; Gómez-Fernández et al., 2012). Sugar could help lower the salt concentration in the unfrozen fraction and prevent solute injury in eutectic freezing (Paniagua-Chávez and Tiersch, 2001). Also, it could be utilized as

an energy source by sperm in terms of glycolysis to support sperm motility and movement (Naing et al., 2010). In this study, the addition of 1% glucose or 2% sucrose into 6% DMSO significantly improved the post-thaw sperm motility. However, no positive effect was revealed at the trehalose concentrations evaluated. This was inconsistent with the studies where trehalose was found to enhance post-thaw sperm motility in some marine mollusc, such as Pacific oysters (Adams et al., 2004, 2008), blacklip pearl oysters (Lyons et al., 2005; Acosta-Salmón et al., 2007; Hui et al., 2011), Greenshell mussels (Smith et al., 2012a) and scallops *Argopecten purpuratus* (Espinoza et al., 2010). In this study, the addition of glucose improved the post-thaw fertilization rate to 83.6 ± 3.7%, which was compatible to the rate (80-85%) recommended by Hone et al. for hatchery production using fresh sperm in this species (Hone et al., 1997). Enhancement in post-thaw sperm quality by glucose has also been found in terrestrial and aquatic species, such as goat and ram (Molinia et al., 1994; Purdy, 2006; Naing et al., 2010), curimba *Prochilodus lineatus* (Viveiros et al., 2009), catfish *Clarias gariepinus* (Steyn, 1993) and common carp *Cyprinus carpio* (Horváth et al., 2003). This enhancement could be explained to some extent by the protective effects of glucose on post-thaw sperm plasma membrane integrity and mitochondrial membrane potential in farmed greenlip abalone that were identified in this study. Increase in the post-thaw sperm fertilization rate by the addition of glucose has also been detected in the rhesus monkey *Macca mulatta* (Si et al., 2006).

In summary, the current study has demonstrated that among the single cryoprotectant solutions evaluated 6% DMSO was optimal for sperm cryopreservation in farmed greenlip abalone. However, DMSO alone could only produce 60% post-thaw sperm fertilization rate. When 6% DMSO was used in combination with 1% glucose, the fertilization rate was improved to > 80%, which is similar to the level recommended

for commercial hatchery productions. Therefore, this study has established the foundation toward the germplasm banking and the application of sperm cryopreservation to enhance the efficiency of genetic improvement programs in this species.

CHAPTER 4 : EFFECTS OF BROODSTOCK AGE AND SPERM COLLECTION TIME OVER A NATURAL SPAWNING PERIOD ON SPERM CRYOPRESERVATION IN FARMED GREENLIP ABALONE*

* This chapter has been submitted as a stand alone manuscript to *Journal of the World Aquaculture Society* as:
Liu, Y., Li, X., Xu, T., Robinson, N., Qin, J., Effect of broodstock age and sperm collection time over a natural spanwing period on sperm cryopreservation in farmed greenlip abalone (under review). Manuscript Number: JWAS-14-264.

4.1 Abstract

This study investigated the effects of broodstock age (2 and 3 years old), and sperm collection time at the beginning, middle and end of a natural spawning period, on the ability of sperm to tolerate cryopreservation in farmed greenlip abalone. The quality of sperm was assessed by motility, fertilization rate, plasma membrane integrity, mitochondrial membrane potential and acrosome integrity. The sperm collected at the middle of a natural spawning period had significantly higher quality than those collected at the beginning and the end of the spawning period in terms of plasma membrane integrity, mitochondrial membrane potential and acrosome integrity of fresh and post-thaw sperm, post-thaw sperm motility and fertilization rate. No significant difference was found between 2 and 3 years old animals in most quality parameters evaluated. The results suggested that sperm collected during the middle of the spawning season should be used for cryopreservation. The efficiency of genetic improvement programs can be further enhanced by using sperm collected from 2-year old abalone which has a similar ability to tolerate cryopreservation as 3 year old counterparts.

Keywords: farmed greenlip abalone, broodstock age, sperm collection time, natural spawning period, sperm cryopreservation

4.2 Introduction

Greenlip abalone *Haliotis laevigata* is the most important farmed abalone species in Australia, accounting for nearly 70% of abalone production in aquaculture (Kube et al., 2007). Genetic improvement techniques, such as selective breeding and hybridization, have been applied to improve production, competitive advantages and long-term sustainable development (Li, 2008). However, these programs are compromised due to asynchronous spawning between male and female abalone (Elliott, 2000). Sperm cryopreservation has been used to address this issue in other species (Gwo et al., 2002; Salinas-Flores et al., 2005) and has been developed in farmed greenlip abalone using a non-programmable freezing technique, resulting in >80% fertilization rate using cryopreserved sperm (Liu et al., 2014b), which is an acceptable level of fertilization in commercial abalone hatcheries (Hone et al., 1997). However, although a very high sperm to egg ratio of 10000:1 was used (Liu et al., 2014b), the fertilization rate (80%) was still lower than what is achieved using sperm collected from wild broodstock (>90%, Zhu et al., 2014). One of the key reasons contributing to these differences may be due to the times when sperm are collected as the published studies have shown that the sperm's ability to tolerant cryopreservation is affected by the timing of sperm collection in relation to the natural spawning period in turbot *Scophthalmus maximus* (Suquet et al., 1998), Atlantic cod *Gadus morhua* and haddock *Melanogrammus aeglefinus* (Rideout et al., 2004). In Pacific oysters *Crassostrea gigas*, Dong et al. (2005c) suggested that the sperm should be collected during the peak spawning period to minimize the influence of seasonal effect on their ability to tolerate cryopreservation (Dong et al., 2005c). In our recent study, 3-year old broodstock were used (Liu et al., 2014b), whereas farmed greenlip

abalone can reach sexual maturity as early as 2 years of age. The rate of genetic gain, and associated economic benefits, would be substantially accelerated if the generation interval could be shortened while maintaining the same accuracy of selection. Moreover, the growth rate of 2-year old abalone has been found to be highly and positively correlated with harvest size of 3-year old animals (Li, 2008). Therefore, if sperm collected from 2-year old, rather than 3 year old, abalone can be cryopreserved for selective breeding, it can lead to substantial time and cost saving, and economic benefits for breeding programs. However, the age of abalone may affect the ability of sperm to tolerate cryopreservation, as such age-dependent effects have been detected in other species, such as Chios sheep (Lymberopoulos et al., 2010) and rooster (Long et al., 2010). The aim of this study was to test the hypothesis that broodstock age and sperm collection time in a natural spawning period can influence the ability of sperm to tolerate cryopreservation. This study would enable us to further improve the reliability of sperm cryopreservation in abalone genetic improvement programs. Sperm from 2- and 3-year old farmed greenlip abalone were collected at the beginning, middle and end of a natural spawning period and their tolerance to cryopreservation was evaluated by measuring post-thaw sperm motility, fertilization rate, plasma membrane integrity, mitochondrial membrane potential and/or acrosome integrity.

4.3 Material and method

4.3.1 Broodstock and gamete collection

The 2- and 3- year old abalone were supplied by SAM Abalone in Port Lincoln, South Australia (SA) over a 3-week interval from 19 September 2013 when the farmed stock were induced to spawn with a standard method until 29 October, a few

days prior to abalone spawning on farm. Both 2- and 3- year old abalone were used on 8 and 29 October whereas only the 3-year old abalone were sampled on 19 September as the 2-year old abalone could not be induced to spawn in the preliminary trial. Abalone of the same age were originally produced from the same spawning trial and more than 20 individuals of each age were collected each time. They were transported by air to the Aquatic Sciences Centre (ASC), South Australian Research and Development Institute, Adelaide, SA. The total body weight was 67.0 ± 13.4 g and 149.6 ± 19.1 g for 2 and 3 years old animals (n = 20), respectively. The animals were acclimated for a maximum of 4 days prior to the spawning induction. During this period, the tanks were on a flow through system with 5-μm filtered seawater at 15.0 ~ 16.0 ºC. The 3 years old females were supplied by the same farm. The methods used for gamete collection and concentration were the same as those described by Liu et al. (2014b).

4.3.2 Experimental procedure

Sperm cryopreservation and quality assessment methods were described by Liu et al. (2014b) unless otherwise specified. Briefly, the pre-cold concentrated sperm were mixed directly with the pre-cold 12% DMSO + 2% glucose at a 1:1 (v:v) ratio and stored on ice for 10 min. The sperm and chemical mixtures were then transferred into 0.25-mL straws which were then placed on a rack 5.2 cm above the surface of liquid nitrogen (LN) in a styrofoam box. The straws were exposed to LN vapour for 10 min before being stored in LN. After at least 12 h storage, the straws were thawed in 60 ^{o}C and 18 ^{o}C seawater baths, respectively. The sperm quality was assessed by sperm motility, fertilization rate, plasma membrane integrity (PMI), mitochondrial membrane potential (MMP) and acrosome integrity (AI). Methods for assessing sperm PMI, MMP and AI by flow cytometery were described by Liu et al. (2014a).

The BD FACSVerse flow cytometer was used with 488-nm argon laser. The FACSuite software provided by the manufacturer was applied to calibrate the instrument settings. A total of 10000 events were analysed for each sample. To collect PMI, MMP or AI data, the density plots were generated with green fluorescence for sperm with intact/functional organelles and with red fluorescence for dead sperm (propidium iodide). Data were analysed using Flowing Software (University of Turku, Finland).

4.3.3 Statistical analysis

Results in this study were presented as mean ± standard deviation (SD). The original percentage data were arcsine transformed before the analysis of variance (ANOVA) with SPSS 20. One-way ANOVA was applied to analyse the results from 3 years old animals. A t-test for independent samples was applied to analyse the results from 2 years old animals and to compare the results between age groups. The Least-Significant Difference (LSD) comparison was used when a significant difference was observed at $P < 0.05$.

4.4 Results

For the 3 year old abalone, the highest post-thaw sperm motility and fertilization rate were found for sperm collected during the middle of the spawning season, significantly higher than those collected at the beginning ($P < 0.05$ for both parameters) and at the end ($P < 0.05$ for the former; $P < 0.01$ for the latter) of the spawning season (Table 4.1). In addition, the post-thaw sperm fertilization rate for the sperm collected at the beginning was significantly higher than that at the end of the spawning season ($P < 0.01$), whereas no significant difference in post-thaw motility was detected between these two sperm collection dates ($P > 0.05$). For 2 year old abalone, both parameters followed the same trends as those found in the 3 year old abalone sperm sample collected during the middle and at the end of the spawning season. Difference in post-thaw motility and fertilization rate between the age groups was not significant in most parameters except for sperm motility at the end of the spawning season.

Table 4.1 Comparisons in post-thaw sperm motilities (%) and fertilization rates (%) between sperm collected at different times during a natural spawning period and between 2 and 3 years old greenlip abalone, $n = 3$.

Sperm collection date	3 years old	2 years old	3 years old	2 years old
	Post-thaw sperm motility (%)		Post-thaw sperm fertilization rate (%)	
Beginning (19/09/2013)	25.3 ± 2.4^{b}		69.2 ± 4.5^{b}	
Middle (8/10/2013)	$30.7 \pm 1.8^{a,A}$	$27.9 \pm 2.1^{a,A}$	$88.5 \pm 2.7^{a,A}$	$79.0 \pm 9.0^{a,A}$
End (29/10/2013)	$24.4 \pm 1.7^{b,A}$	$19.9 \pm 2.0^{b,B}$	$34.3 \pm 10.4^{c,A}$	$24.2 \pm 6.8^{b,A}$

Different lowercase letters within each age group indicate significant difference between sperm collected at different times in a natural spawning period ($P < 0.05$). Different capital letters within the same sperm collection date indicate significant difference between age groups ($P < 0.05$).

The PMI, MMP and AI values in fresh sperm were significantly higher in samples collected during the middle than those collected at the beginning and the end of the spawning season in 3 years old abalone ($P < 0.05$) (Table 4.2). No significant difference was found between the sperm collected at the beginning and the end of the spawning season ($P > 0.05$). In 2 year old abalone, PMI and MMP values were significantly higher in the sperm collected during the middle than at the end of the spawning season ($P < 0.05$), whereas the difference in AI was not significant ($P > 0.05$). No significant difference in these parameters was found between age groups on the respective sperm collection dates ($P > 0.05$).

Table 4.2 Comparisons in plasma membrane integrity, mitochondrial membrane potential and acrosome integrity values in fresh sperm collected from 2 and 3 years old greenlip abalone at different time during the spawning season, $n = 3$.

Sperm collection date	Sperm quality assessment		
	Parameters	3 years old	2 years old
Beginning (19/9/2013)		70.7 ± 2.6^{b}	
Middle (8/10/2013)	PMI (%)	$80.5 \pm 1.6^{a,A}$	$79.8 \pm 2.4^{a,A}$
End (29/10/2013)		$71.2 \pm 5.1^{b,A}$	$69.8 \pm 3.9^{b,A}$
Beginning (19/9/2013)		70.1 ± 3.1^{b}	
Middle (8/10/2013)	MMP (%)	$78.8 \pm 1.9^{a,A}$	$77.3 \pm 2.1^{a,A}$
End (29/10/2013)		$73.1 \pm 3.0^{b,A}$	$72.6 \pm 1.3^{b,A}$
Beginning (19/9/2013)		76.8 ± 1.9^{b}	
Middle (8/10/2013)	AI (%)	$82.3 \pm 3.0^{a,A}$	$79.0 \pm 5.3^{a,A}$
End (29/10/2013)		$72.4 \pm 1.2^{b,A}$	$69.4 \pm 3.0^{a,A}$

Different lowercase letters within each age group indicate significant difference between sperm collected at different times in a natural spawning period ($P < 0.05$). Different capital letters within the same sperm collection date indicate significant difference between age groups ($P < 0.05$).

Similar trends were observed for the post-thaw sperm PMI, MMP and AI results as fresh sperm (Table 4.3). In general, the values in sperm collected during the middle of spawning season were significantly higher than those collected at other times. At the same collection time, differences in post-thaw sperm PMI, MMP and AI values were not significant, except for AI values in the sperm collected during the middle of the spawning season, whereas these values were higher in sperm of 3 year old abalone than those of 2 year old abalone.

Table 4.3 Comparisons in plasma membrane integrity, mitochondrial membrane potential and acrosome integrity values in post-thaw sperm collected from 2 and 3 year old greenlip abalone at different time during the spawning season, $n = 3$.

Sperm collection date	Sperm quality assessment		
	Parameters	3 years old	2 years old
Beginning(19/9/2013)		20.8 ± 4.0^{ab}	
Middle (8/10/2013)	PMI (%)	$24.4 \pm 3.6^{a,A}$	$24.3 \pm 1.9^{a,A}$
End (29/10/2013)		$14.8 \pm 2.1^{b,A}$	$15.8 \pm 3.2^{b,A}$
Beginning (19/9/2013)		34.8 ± 1.2^{a}	
Middle (8/10/2013)	MMP (%)	$44.7 \pm 3.9^{b,A}$	$35.2 \pm 5.4^{a,A}$
End (29/10/2013)		$28.3 \pm 1.5^{c,A}$	$25.9 \pm 1.7^{A,b}$
Beginning (19/9/2013)		41.6 ± 0.5^{a}	
Middle (8/10/2013)	AI (%)	$45.6 \pm 2.1^{b,A}$	$37.9 \pm 4.1^{a,B}$
End (29/10/2013)		$27.3 \pm 0.9^{c,A}$	$25.6 \pm 5.5^{b,A}$

Different lowercase letters within each age group indicate significant difference between sperm collected at different times in a natural spawning period ($P < 0.05$). Different capital letters within the same sperm collection date indicate significant difference between age groups ($P < 0.05$).

4.5 Discussion

This study investigated the tolerance to cryopreservation damage in sperm collected at different times over the natural spawning period in 2 and 3 year old greenlip abalone. The results showed that the sperm collected during the middle of the natural spawning period had the highest ability to tolerate the cryopreservation processes, whereas little difference was found between 2 and 3 year old stocks. Therefore, it is possible to use the sperm of 2 year old abalone for cryopreservation, which could be beneficial for genetic improvement programs by reducing the generation interval for a selective breeding program.

Unlike oysters, and most finfish species where sperm are collected using the strip spawning method, sperm of abalone species are collected through the natural spawning methods (Marshall and McPherson, pers. comm.). In this study, more than half of the individuals were successfully induced to spawn each time. Although a larger volume of concentrated sperm was collected from 3 than 2 year old abalone individuals, the sperm concentration of 10^9/mL was achieved in abalone of both age groups.

High fresh sperm quality is essential to the success of cryopreservation (D'Alessandro and Martemucci 2003; Lukaszewicz et al. 2003). Studies in livestock and fish species have demonstrated that fresh sperm quality is affected by the sperm collection time during a spawning period, which reflects their ability to tolerate cryopreservation (Janett et al., 2003; Rouxel et al., 2008; Koivisto et al., 2009; Coloma et al., 2011). In Port Lincoln, South Australia, the farmed greenlip abalone mature at 2 years of age and spawn from mid September to late October each spawning season (Marshall, per. comm.). In this study, the quality of sperm collected

in the middle of this period was better than that collected at the beginning and at the end, as the former showed higher PMI, MMP and AI values than the latter. This result is in agreement with that in Atlantic cod *Gadus morhua* (Rouxel et al., 2008), red porgy *Pagrus pagrus* (Mylonas et al., 2003) and Atlantic halibut *Hippoglossus hippoglossus* (Babiak et al., 2006), where the highest quality sperm was also found in the middle of the spawning season. The highest quality of fresh sperm was from that collected in the middle of spawning season in this study, and sperm collected at this time showed the highest post-thaw sperm motility, fertilization rate and PMI, MMP and AI values. Similar findings were also reported in Atlantic cod and haddock (Rideout et al., 2004), Iberian ibex *Capra pyrenaica* (Coloma et al., 2011), Franches-Montagnes and Warmblood stallion (Janett et al., 2003), Leccese ram (D'Alessandro and Martemucci, 2003) and *Bos indicus* and *Bos Taurus* bulls (Koivisto et al., 2009) where a higher sperm ability to tolerate cryopreservation was found in sperm collected at a certain time of a natural spawning period. In the current study, most of the parameters evaluated were not significantly different between the abalone in the two age groups at respective spawning times. These results do not agree with the studies where sperm collected from older broodsotck produce a better ability to tolerate cryopreservation in comparison with their younger counterparts, such as found for rams (Lymberopoulos et al., 2010) and white Italian ganders *Anser anser* (Lukaszewicz et al., 2003). However, caution should be taken as there was only 1 year difference in age between the two age groups used in this study. It is anticipated that the use of sperm from younger broodstock would not only improve the genetic gains in selective breeding programs, but also reduce the time and cost for broodstock maintenance and minimise the potential risks from disease outbreak and natural disasters.

CHAPTER 5 : GREENLIP ABALONE (*HALIOTIS LAEVIGATA* DONOVAN, 1808) SPERM CRYOPRESERVATION USING A PROGRAMMABLE FREEZING TECHNIQUE AND TESTING THE ADDITION OF AMINO ACID AND VITAMIN*

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5.1 Abstract

This study investigated factors key to the development of sperm cryopreservation in the greenlip abalone *Haliotis laevigata* using a programmable freezing technique, including (1) permeable cryoprotectant agent (CPA) selection; (2) cooling rate; (3) endpoint temperature; (4) thawing temperature; (5) sperm to egg ratio; and (6) sugar, vitamin and amino acid supplementation, using sperm motility, fertilization rate, plasma membrane integrity, mitochondrial membrane potential (MMP) or acrosome integrity (AI) as quality assessment indicators. Results showed that among the permeable CPAs evaluated 10% dimethyl sulfoxide (DMSO) was the most suitable for greenlip abalone sperm cryopreservation. The highest post-thaw sperm motility was achieved with the sperm being frozen at a cooling rate of -5 °C/min to -30 ºC from 0 ºC and thawed and recovered in 40 ºC and 18 ºC seawater baths respectively. The addition of sugars in 10% DMSO did not significantly improve the post-thaw sperm motility and fertilization rate. The addition of 0.6% glycine, 0.2% taurine or 0.02% L-ascorbic acid, on the other hand, significantly improved the post-thaw sperm motility. However, only the addition of 0.6% glycine improved the post-thaw sperm fertilization rate, which was further confirmed by the improvement of the post-thaw sperm MMP and AI through flow cytometry analysis.

Keywords: greenlip abalone, *Haliotis laevigata*, programmable freezing technique, amino acids, vitamins

5.2 Introduction

Greenlip abalone *Haliotis laevigata* Donovan is a highly valuable species that accounts for about 70% of abalone aquaculture production in Australia (Kube et al., 2007). To ensure the long-term sustainable development of the industry, genetic improvement programs using selective breeding have been initiated (Kube et al., 2007; Li, 2008; Robinson et al., 2010). However, it is challenging to mate desired broodstock combination due to asynchronous spawning between males and females (Elliott, 2000) and short longevity of the gametes collected. There have also been problems with adequately backing up genetically improved stock so that there is a risk of losing them when disease outbreaks and natural disasters occur.

Sperm cryopreservation could offer opportunities to address the above issues (Chao, 1996; Cabrita et al., 2010) and has been routinely applied in breeding programs in terrestrial species, such as stallions (Janett et al., 2003) and buffalo bulls (Bhakat et al., 2011). Although both programmable freezing and liquid nitrogen (LN) vapour techniques are used in sperm cryopreservation, the former has been found being more reliable and resulting in better post-thaw sperm qualities (such as motility, plasma membrane integrity, etc) in comparison with the latter in dog (Rota et al., 2005), human (Stanic et al., 2000), stallion (Clulow et al., 2008) and honey bee *Api mellifera* (Hopkins and Herr, 2010). Furthermore, the sperm quality parameters have also been proven to positively correlate with sperm fertilizing ability in marine mollusc (Lyons et al., 2005; Paniagua-Chavez et al., 1998, 2006, Vitiello et al., 2011). These advantages often make the programmable freezing technique the preferential option for the application of cryopreservation in breeding programs and semen cryobank establishment (McLaughlin et al., 1990; Conget et al., 1996; Stanic et al.,

2000; Clulow et al., 2008). Cryopreservation of greenlip abalone sperm has been investigated using LN vapour technique in our previous study, with 80% post-thaw sperm fertilization rate being produced at a sperm to egg ratio of 10000:1 (Liu et al., 2014b), which is much higher than when fresh sperm is used (200:1). This means that a large quantity of cryopreserved sperm will be needed for abalone hatchery production or selective breeding programs to manage differences in response to cryopreservation stresses among abalone. Therefore, the programmable freezing technique was evaluated in this study in an anticipation to improve the reliability and efficiency of the existing cryopreservation protocol.

Information from livestock and fish species has shown that the addition of sugars, amino acids and vitamins is a good strategy to improve the post-thaw sperm quality (He and Woods, 2003; Li et al., 2003; El-Sheshtawy et al., 2008; Khalili et al., 2010; Martínez-Páramo et al., 2012; Memon et al., 2013). Post-thaw sperm motility, the integrity of post-thaw sperm membrane and acrosome, and the mitochondrial membrane potential have been improved significantly by the addition of sugars in ram (Jafaroghli et al., 2011) and boars (Gómez-Fernández et al., 2012), amino acids in cynomolgus monkey *Macaca fascicularis* (Li et al., 2003), buffalo bull (El-Sheshtawy et al., 2008), striped bass *Morone saxatilis* (He and Woods, 2004) and moghani ram (Khalili et al., 2010), and vitamins in boer goat (Memon et al., 2013) and sea bass *Dicentrarchus labrax* (Martínez-Páramo et al., 2012). In marine mollusc, sugar supplementation has been reported with inconsistent results. Glucose has shown the positive role in farmed greenlip abalone (Liu et al., 2014a), but not in its wild counterpart and small abalone *H. diversicolor supertexta* (Gwo et al., 2002; Zhu et al., 2014). Although vitamins and amino acids have been supplemented in the cryoprotective medium in a few oyster studies (Zell et al., 1979; Yankson and Moyse,

1991; Zhang et al., 2012), these supplements have not been investigated in abalone sperm cryopreservation. In this study, factors affecting sperm cryopreservation using the programmable freezing technique were evaluated in greenlip abalone. The addition of sugars, amino acids and vitamins was also assessed to determine whether their supplementation could further enhance post-thaw sperm qualities.

5.3 Material and methods

5.3.1 Animals and gamete preparation

The animals were provided during the natural spawning season by the SAM Abalone in Port Lincoln, South Australia (SA) and Kangaroo Island Abalone on Kangaroo Island, SA and transported by air to the Aquatic Sciences Centre, South Australian Research and Development Institute (SARDI). The animals were about 3 years old and 122.0 ± 8.9 g in total body weight. After acclimation for at least 5 days, abalone with large and swollen gonads were selected for spawning induction with a combination of temperature shock and ultraviolet irradiated seawater (Liu et al., 2014b). Concentrated sperm were pooled from at least three males at an equal volume. The sperm were diluted to 1.6×10^8 mL^{-1} with 5 μm filtered seawater and stored on ice for the subsequent experiments within 1.5 h. Eggs were gently poured through a 300 μm sieve and obtained on a 90 μm sieve underneath. The collected eggs were gently rinsed with 5 μm filtered seawater and then washed into a settlement beaker. After 15 min the eggs on the bottom were transferred into another container and mixed gently by hand. One millilitre egg suspension was taken and diluted 100 times before a 1 mL subsample was used to account the egg density under a microscope. This estimation was repeated three times per collection. The egg density was then standardised to 1×10^3 mL^{-1}. The eggs used in this study were never

older than 2 h post spawning.

5.3.2 Chemicals

Dimethyl sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG), sucrose, glucose, trehalose, glycine, taurine and L-ascorbic acid were in AR grade (Sigma-Aldrich Pty Ltd, St Louis, MO, USA). Double concentration stock solutions were prepared in 5 μm filtered seawater such that each chemical mixed with sperm at a 1:1 ratio (v/v) yielded the final concentration required.

A LIVE/DEAD sperm viability kit (L-7011) for plasma membrane integrity (PMI) evaluation and LysoTracker green DND-26 (LYSO-G) kit (L-7526) for acrosome integrity (AI) evaluation were purchased from Invitrogen (Eugene, Oregon, USA). Rhodamine 123 (Rh 123) for mitochondrial membrane potential (MMP) evaluation and propidium iodide (PI) used for AI and MMP evaluation were also purchased from Sigma-Aldrich Pty Ltd. The working solutions of these fluorescent agents were prepared according to Liu et al. (2014b).

5.3.3 Equipment

A CL863 programmable freeze controller (Cryologic, Mulgrave, Victoria, Australia) was used. Straws (Minitube, Germany) were placed into a cryochamber (model: CC23F) with a lid on top and frozen by LN. The temperature was regulated by an electronic device inside the cryochamber in accordance with the protocol set and monitored with the CryoGenesis software (V5) from the Cryologic.

A thawing and a recovery seawater bath were used in this study. The required temperatures (30 to 80 ºC) in the thawing bath were achieved by mixing the ambient and boiled seawater. The temperature required in the recovery bath (18 ºC) was achieved by mixing the ambient and cold seawater.

The BD FACSVerse flow cytometer was used with 488 nm argon laser. The FACSuite software provided by the manufacturer was applied to calibrate the instrument settings.

5.3.4 Sperm quality evaluation methods

The motility rate was determined by counting the number of active sperm out of 100 by two independent observers after sperm were diluted to 2×10^6 sperm/mL. Sperm moving forward progressively were counted as active sperm while those vibrating or not moving at all were counted as dead sperm (Gwo et al., 2002). To standardize across the experiments, the percentage of fresh sperm motility was set to 100% and the post-thaw sperm motility value was calculated as a percentage of the fresh sperm motility.

The fertilization assessment was conducted in 10 mL tubes. During fertilization, the concentrated eggs were taken using a pipette to reach the desired sperm to egg ratio. After mixing with sperm for 10 min, the eggs were washed gently on a 90 μm sieve by 5 μm filtered seawater prior to being cultured in a 500 mL container. The fertilization rate was determined microscopically 4 h post-fertilization by counting the number of fertilized eggs out of a sample of 100. Eggs with two or multiple cells were counted as fertilized eggs and the rest was counted as unfertilized eggs. Controls were established using a sperm to egg ratio of 200:1.

For evaluation of plasma membrane integrity (PMI), mitochondrial membrane potential (MMP) and acrosome integrity (AI), thawed sperm were diluted to 2×10^6 sperm/mL and 1 mL of diluted sperm was then stained fluorescently prior to flow cytometric analysis. For PMI and MMP assessments, 100 μL SYBR14 and Rh123 were added for 20 min, respectively, and then 100 μL PI for a further 10 min. For AI

analysis, 5 µL LYSO-G was added for 30 min, and then 9 µL PI for a further10 min. All staining was carried out at the room temperature (Liu et al., 2014b). A total of 10000 events were read per sample. For plasma membrane integrity data, density plots were generated with green fluorescence for sperm with intact plasma membrane (SYBR 14) and red fluorescence for dead sperm (PI). For mitochondrial membrane potential data, density plots were generated with green fluorescence for sperm with functional mitochondria (Rh 123) and red fluorescence for dead sperm (PI). For acrosome integrity data, density plots were generated with green fluorescence for sperm with intact acrosome (LYSO-G) and red fluorescence for dead sperm (PI). Data were analysed using the Flowing Software (University of Turku, Finland).

5.3.5 Experiments

5.3.5.1 Effects of different permeable CPAs on post-thaw sperm motility

Ten minutes equilibration on ice and 8% DMSO, EG and PG were selected in this experiment according to the results published by Liu et al. (2014b) in the same species. After equilibration, the sperm and cryoprotectant solution mixtures were transferred into 0.25 mL straws, which were then put into the programmable freeze controller. The straws were frozen at -5 °C/min from 0 °C to -50 °C (endpoint temperature) before being transferred into LN directly for at least 12 h storage. The straws were thawed in a 60 °C seawater bath until ice melted in 5 s and recovered in an 18 °C seawater bath. A subsample was collected to assess motility.

This and all the subsequent experiments were repeated three times using different sperm pools and each treatment was in three replicates.

5.3.5.2 Effects of cooling rate and DMSO concentration on post-thaw sperm motility

In the previous experiment, sperm cryopreserved in DMSO achieved the highest

post-thaw motility. In this experiment, DMSO at different concentrations was applied to evaluate the effects of cooling rates at -3, -5 and -7 °C/min on post-thaw sperm motility. The other procedures were the same as section *5.3.5.1*.

5.3.5.3 Effects of thawing temperature on post-thaw sperm motility

The cooling rate of -5 °C/min with 10% DMSO achieved the highest post-thaw sperm motility and was used in this and subsequent experiments. In this experiment different thawing temperatures were assessed, 30, 40, 50, 60, 70 and 80 °C, for 8, 7, 6, 5, 4, and 3 s, respectively. The other procedures were the same as section *5.3.5.2*.

5.3.5.4 Effects of endpoint temperature on post-thaw sperm motility

The highest post-thaw sperm motility was achieved at 40 °C thawing temperature and this temperature was used in this and subsequent experiments. In this experiment, endpoint temperatures of -20, -30, -40, -50, -60, -70, -80 and -90 °C were evaluated. The other procedures were the same as section *5.3.5.3*.

5.3.5.5 Assessment of types and concentrations of sugar, amino acid and vitamin on post-thaw sperm motility and fertilization rate

The treatment (10% DMSO, -5 °C/min freezing rate, -30 °C endpoint temperature and 40 °C thawing temperature) produced the highest post-thaw sperm motility in the previous experiments and was used in this experiment to evaluate the effects of the addition of sugars (1, 2, 3, 4 or 5% sucrose, glucose or trehalose), amino acids (0.4, 0.6, 0.8 or 1.0% glycine and 0.05, 0.1, 0.2 or 0.4% taurine) and vitamins (0.01, 0.02 or 0.04% L-ascorbic acid) on post-thaw sperm motility. Further evaluation of fertilization rates was made by comparing 10% DMSO and its combination with 4% sucrose, 0.6% glycine, 0.2% taurine and 0.02% L-ascorbic acid at a sperm to egg ratio of 10000:1, 20000:1 or 40000:1. The other procedures were the same as section

5.3.5.4.

5.3.5.6 Assessment of post-thaw sperm plasma membrane integrity (PMI),mitochondrial membrane potential (MMP) and acrosome integrity (AI)

The highest post-thaw sperm fertilization rate was achieved when sperm were cryopreserved in 10% DMSO + 0.6% glycine. In this experiment, post-thaw sperm PMI, MMP and AI were assessed comparing the use of 10% DMSO and its combination with 0.6% glycine by flow cytometer.

5.3.6 Statistical analysis

Results in this study were presented as mean ± standard deviation (SD). The original data were arcsine transformed before the analysis with SPSS 20 (SPSS lnc., Chicago, IL, USA). A t test for independent samples was applied to analyse the impacts of the addition of 0.6% glycine in *section 5.3.5.6*. One-way analysis of variance (ANOVA) was applied to analyse the experiments on permeable CPA protection, cooling rate, thawing temperature, endpoint temperature, amino acid and vitamin on post-thaw sperm motility. Two-way ANOVA was applied to analyse the experiments on cooling rate and DMSO concentration, type and concentration of sugar, and cryoprotectant solution and sperm to egg ratio on post-thaw sperm motility or fertilization rate. When the significant interaction was found between the two factors, the main effects were tested. The Least-Significant Difference (LSD) comparison was used when a significant difference was observed. Differences between means were considered significantly at $P < 0.05$. Power analysis by G*Power software (V3; Heinrich Heine Universitat Dusseldorf, North Rhine-Westphalia, Germany) was conducted to predict the required sample size given the mean difference and SD observed on our preliminary results evaluating the “effect of cryoprotectant toxicity

on sperm motility" and "effect of thawing temperature on post-thaw sperm motility" as these two factors have been shown to influence the sperm motility in published marine mollusc studies. The results showed that for an ability to detect a difference among four groups within cryoprotectant toxicity (Cohen *f*, 2.581; SD, 3.5; groups with equal size) or five groups within thawing temperature (Cohen *f*, 2.499; SD, 1.7; groups with equal size) with α was set at 0.05 and power was set at 0.9, a sample size of 3 replicates in each group was enough.

5.4 Results

5.4.1 Effects of different permeable CPAs on post-thaw sperm motility

Eight percent DMSO produced the highest post-thaw sperm motility which was significantly higher than EG and PG at the same concentration (Fig. 5.1).

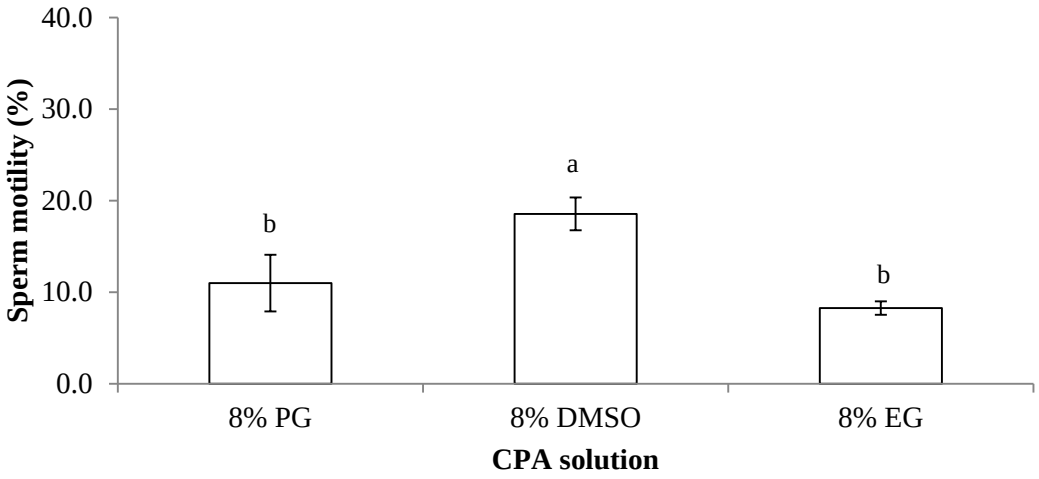

Figure 5-1 Post-thaw sperm motilities (%; relative to control) after being cryopreserved in 8% PG, DMSO or EG, $n = 3$. Bars with different letters differ significantly ($P < 0.05$).

5.4.2 Effects of cooling rate and DMSO concentration on post-thaw sperm motility

Significant interaction ($P < 0.05$) between cooling rate and DMSO concentration was found. Within each cooling rate treatment, the post-thaw sperm motility increased with the increase in DMSO concentrations (Fig. 5.2). Within each DMSO concentration, the post-thaw sperm motility decreased with the increase in cooling rate at the two lower DMSO concentrations, whereas no significant difference ($P > 0.05$) was found between cooling rates in 10% DMSO. The highest post-thaw sperm motility was produced at -5 °C/min in 10% DMSO, even though no significant

differences ($P > 0.05$) were found with other cooling rates at this DMSO concentration (Fig. 5.2).

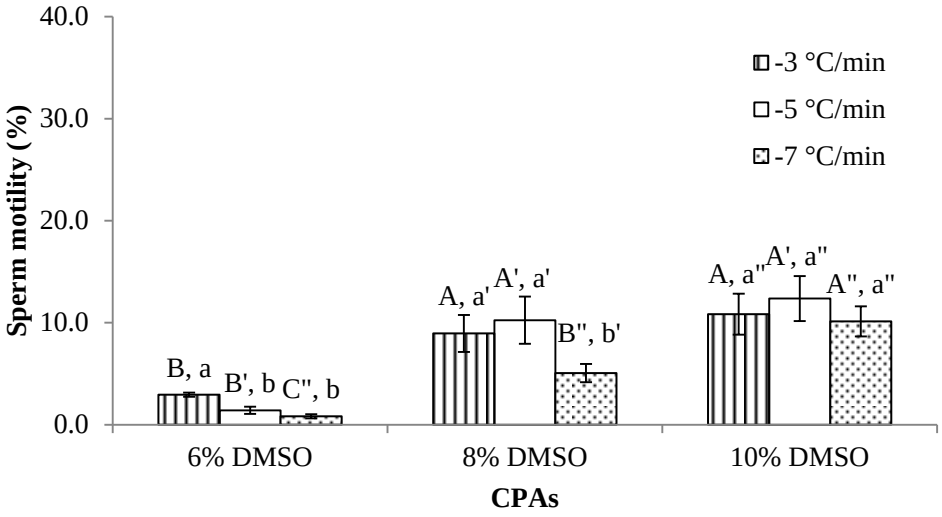

Figure 5-2 Post-thaw sperm motilities (%; relative to control) after being frozen at different cooling rates in 6, 8 or 10% DMSO in a programmable freeze controller, *n* = 3. Different capital letters within each cooling rate indicate significant differences between DMSO concentrations. Different lowercase letters within each DMSO concentration indicate significant differences between cooling rates ($P < 0.05$).

5.4.3 Effects of thawing temperature on post-thaw sperm motility

A thawing temperature of 40 °C achieved the highest post-thaw sperm motility of 18.8 ± 0.6%, which was significantly higher than the other temperatures (Fig. 5.3).

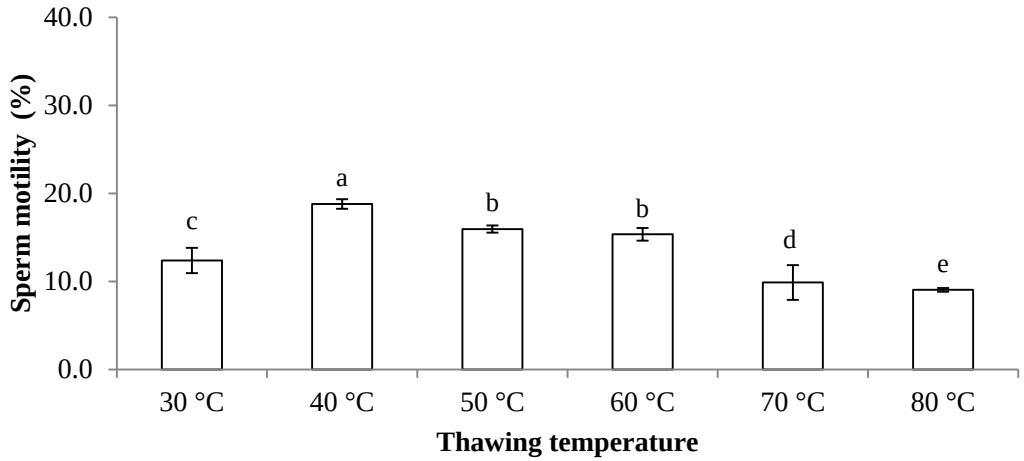

Figure 5-3 Sperm motilities (%; relative to control) after being thawed at different temperatures in a programmable freeze controller, $n = 3$. Different letters indicate significant differences ($P < 0.05$).

5.4.4 Effects of endpoint temperature on post-thaw sperm motility

The post-thaw sperm motility increased as the endpoint temperatures increased, reaching the highest value at -30 ℃ which was significantly higher than others ($P < 0.01$, Fig. 4). No post-thaw motility was observed at a -20 ℃ endpoint temperature.

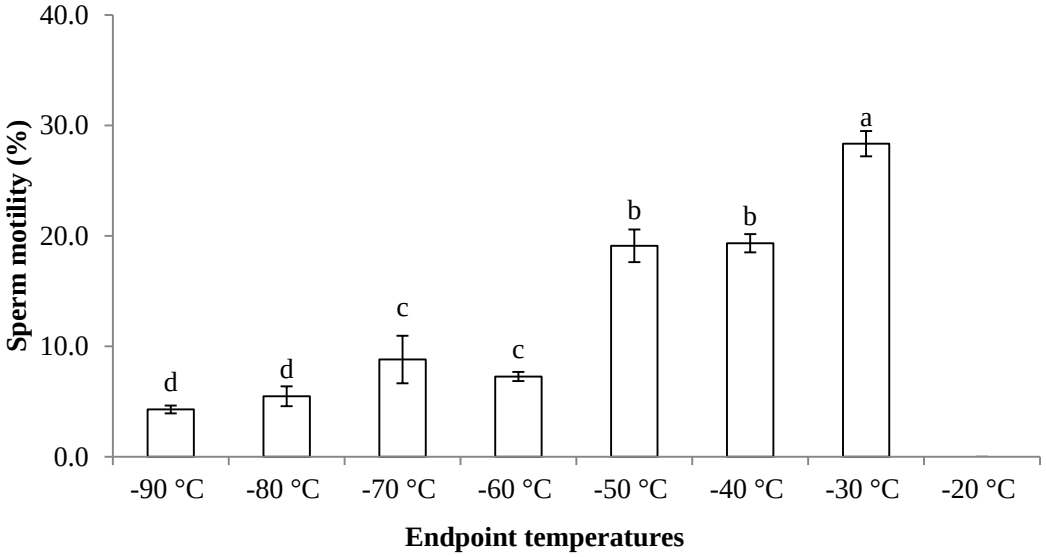

Figure 5-4 Post-thaw sperm motilities (%; relative to control) after being frozen to different endpoint temperatures in a programmable freeze controller before being transferred into LN, n = 3. Bars with different letters differ significantly ($P < 0.05$).

5.4.5 Assessment of types and concentrations of sugar, amino acid and vitamin on post-thaw sperm motility and fertilization rate

Fig. 5.5 showed that only the addition of 4% sucrose in 10% DMSO significantly improved the post-thaw sperm motilities. The addition of other sugar with different concentrations either did not improve or decreased post-thaw sperm motilities.

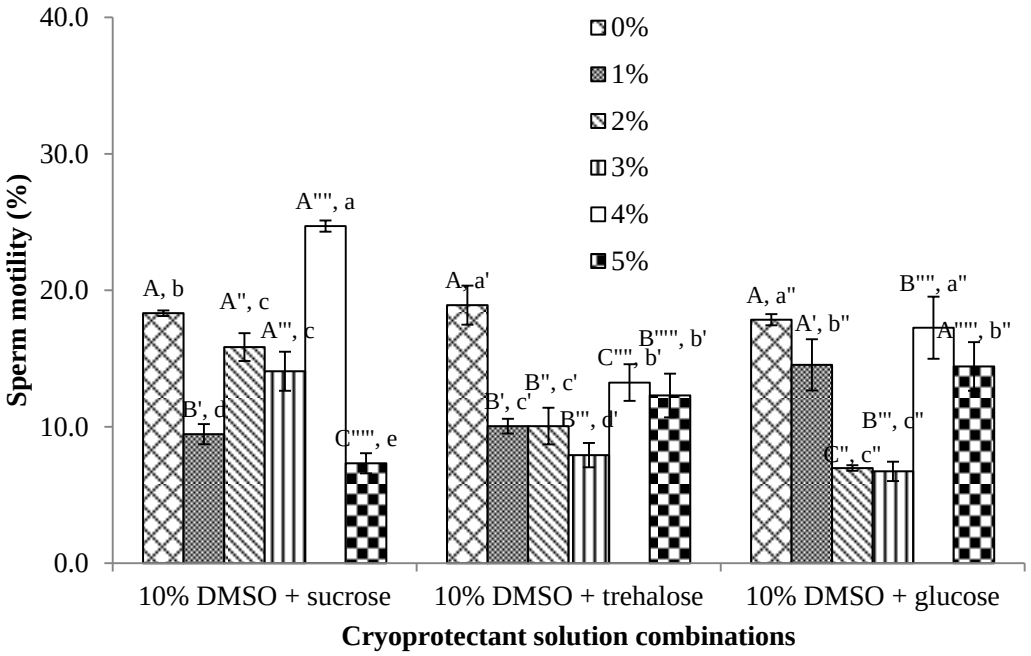

Figure 5-5 Comparison of post-thaw sperm motilities (%; relative to control) after being frozen in 10% DMSO or its combination with different types and concentrations of sugar, $n = 3$. Bars with different capital letters within each sugar concentration (+ 10% DMSO) differ significantly between different sugar types ($P < 0.05$). Bars with different lowercase letters within each sugar type (+ 10% DMSO) differ significantly between different sugar concentrations ($P < 0.05$).

With respect to amino acid and vitamin treatments, the concentration resulting in the highest post-thaw sperm motility in each chemical was 10% DMSO + 0.6% glycine (Fig.5.6), 10% DMSO + 0.2% taurine (Fig. 5.7) and 10% DMSO + 0.02% L-ascorbic acid (Fig. 5.8).

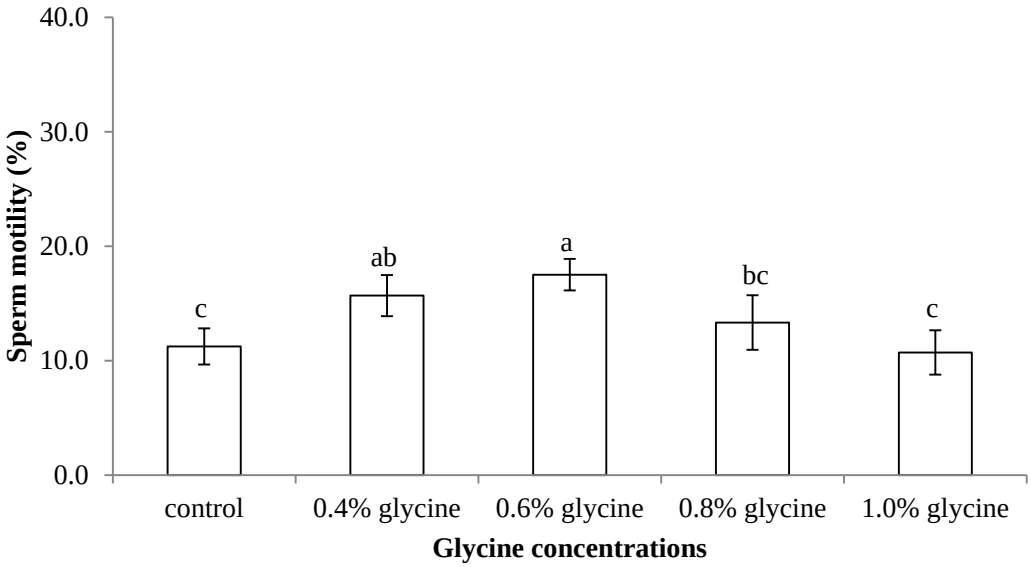

Figure 5-6 Comparisons of post-thaw sperm motilities (%; relative to control) between 10% DMSO (control) and its combination with different concentrations of glycine, $n = 3$. Bars with different letters differ significantly ($P < 0.05$).

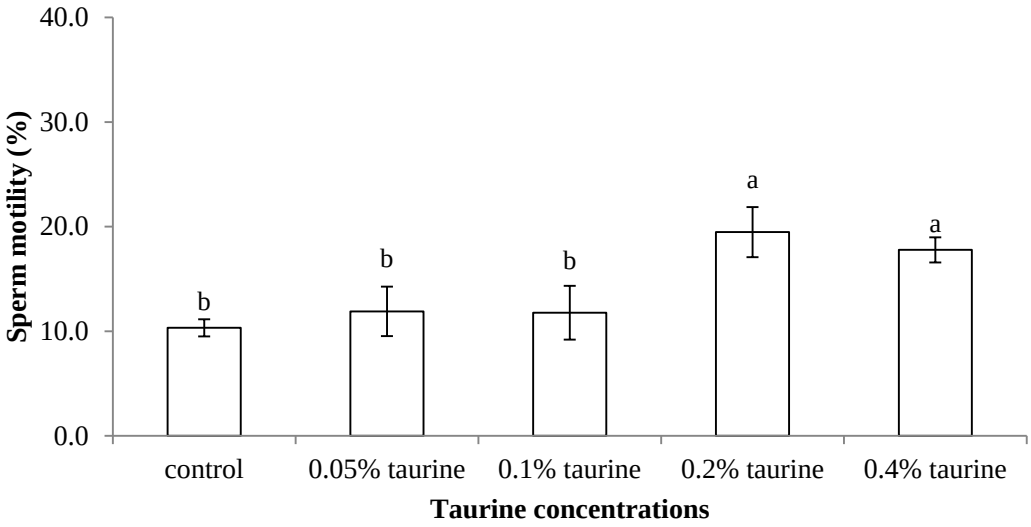

Figure 5-7 Comparisons of post-thaw sperm motilities (%; relative to control) between 10% DMSO (control) and its combination with different concentrations of taurine, $n = 3$. Bars with different letters differ significantly ($P < 0.05$).

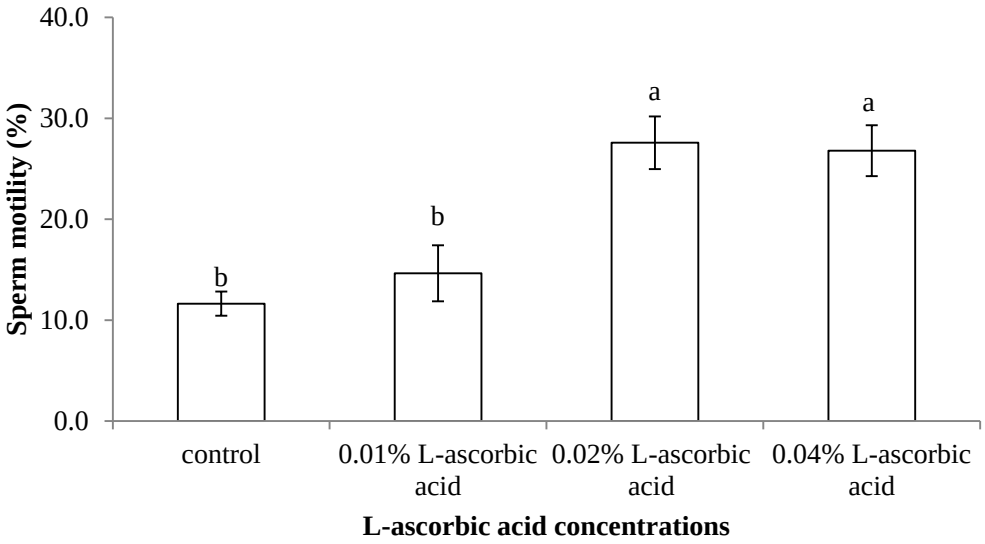

Figure 5-8 Comparisons of post-thaw sperm motilities (%) between 10% DMSO (control) and its combination with different concentrations of L-ascorbic acid, $n = 3$. Bars with different letters differ significantly ($P < 0.05$).

Further comparison on post-thaw sperm fertilization rates in these three treatments showed that the post-thaw fertilization rates between sperm cryopreserved in 10% DMSO and 10% DMSO + 4% sucrose were not significantly different from each other at the sperm to egg ratio of 40000:1, with both being significantly lower than the control (Fig. 5.9). However, 10% DMSO + 0.6% glycine achieved the highest rates at the sperm to egg ratio of 40000:1, which was similar to the control (sperm to egg ratio of 200:1; $P > 0.05$) (Fig. 5.9).

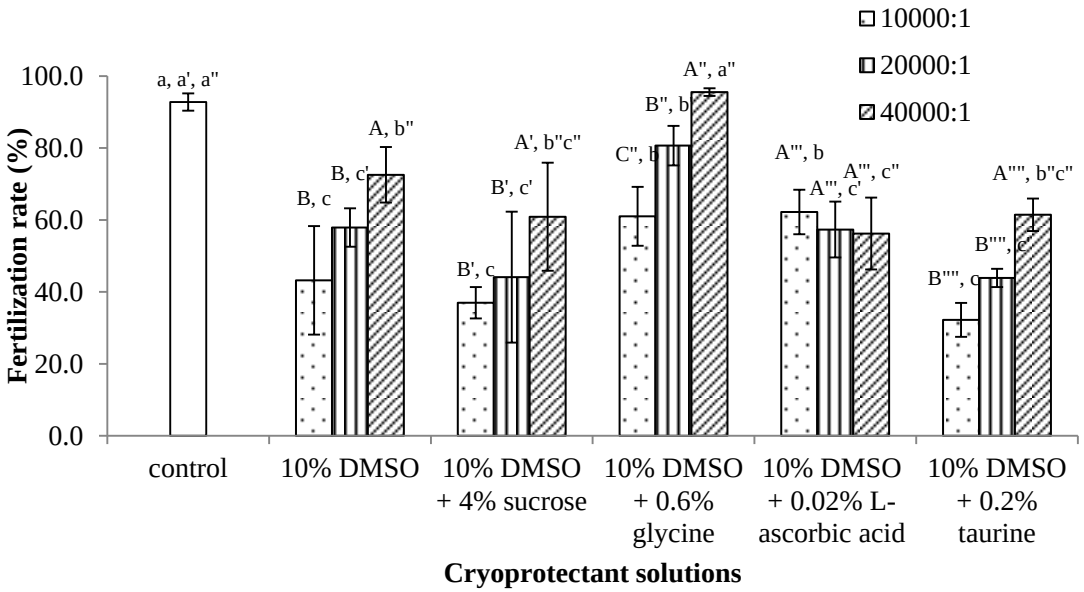

Figure 5-9 Effects of 10% DMSO and its combination with 4% sucrose, 0.6% glycine, 0.02% L-ascorbic acid or 0.2% taurine on post-thaw sperm fertilization rate (%) at different sperm to egg ratios, $n = 3$. Bars with different capital letters within each cryoprotectant solution differ significantly between different sperm to egg ratios ($P < 0.05$). Bars with different lowercase letters within each sperm to egg ratio differ significantly between different cryoprotectant solutions ($P < 0.05$).

5.4.6 Assessment of post-thaw sperm plasma membrane integrity (PMI), mitochondrial membrane potential (MMP) and acrosome integrity (AI)

Post-thaw sperm MMP and AI was significantly improved when 0.6% glycine was added in 10% DMSO. However, the PMI was not affected by the addition of glycine (Table 5.1).

Table 5.1 Comparison of plasma membrane integrity (PMI), mitochondrial membrane potential (MMP) and acrosome integrity (AI) between sperm cryopreserved in 10% DMSO and 10% DMSO + 0.6% glycine, $n = 3$.

CPA combinations	Sperm component and organelle		
	PMI	MMP	AI
10% DMSO	21.6 ± 2.6%[a]	13.9 ± 2.8%[b]	19.8 ± 2.3%[b]
10% DMSO + 0.6% glycine	23.4 ± 3.2%[a]	20.4 ± 2.7%[a]	26.6 ± 1.6%[a]

Different letters within each assessment indicate significant difference ($P < 0.05$).

5.5 Discussion

This study has optimized the parameters required in the programmable freezing technique for greenlip abalone sperm cryopreservation. The post-thaw sperm fertilization rate of about 70% was achieved by using 10% DMSO as a cryoprotectant solution and could be further improved to above 90% after the addition of 0.6% glycine. The reason for this improvement could partially be attributed to the improvement in the post-thaw sperm mitochondrial membrane potential and acrosome integrity, suggesting a positive role of glycine in sperm cryopreservation in this species.

During cryopreservation an ideal CPA should have low toxic effects and at the same time should give high cryoprotective ability to sperm (Ieropoli., et al., 2004; Lyons et al., 2005). Among the permeable CPAs evaluated in this study, DMSO showed the best cryoprotective effects. Further evaluation revealed that 10% DMSO was the best concentration for greenlip abalone sperm cryopreservation when used with a programmable freezing technique. This result (10% DMSO) was similar to that reported in small abalone (Gwo et al., 2002), Pacific oysters (Gwo et al., 2003), pearl oysters *Pinctada martensii* (Wang et al., 2006) and scallops *Patinopecten yessonesis* and *Chlamys farreri* (Yang et al., 1999). However, this concentration was higher than those found by the previous studies in this species (Liu et al., 2014b; Zhu et al., 2014), and in blacklip abalone *H. rubra* (Liu et al., in press b) and blacklip pearl oysters *P. margaritifera* (Lyons et al., 2005). On the contrary, it was lower than those reported in macha surf clam *Mesodesma donacium* (Dupré and Guerrero, 2011) and greenshell mussels *Perna canaliculus* (Smith et al., 2012a). The discrepancy in optimal DMSO concentration between this current study and those of Zhu et al.

(2014) and Liu et al. (2014b) may be due to differences in freezing techniques. Programmable freezing technique was used in this study, whereas LN vapour technique was applied in the studies by Zhu et al. (2014) and Liu et al. (2014b). A similar phenomenon has also been revealed in Pacific oysters (Ieropoli et al., 2004; Adams et al., 2008), rainbow trout *Oncorhynchus mykiss* (Lahnsteiner et al., 1995; Conget et al., 1996) and paddlefish *Polyodon spathula* (Linhart et al., 2006; Horváth et al., 2003), where a higher CPA concentration was required for the programmable freezing technique. However, reasons for higher CPA concentration requirement in the programmable than non-programmable freezing techniques were unclear.

Cooling rate is of importance as an optimal selection could balance the effects from the formation of intracellular ice and the increase in solute concentrations, leading to maximally maintain the sperm quality (Gao and Critser, 2000; Chao and Liao, 2001; Ieropoli et al., 2004; Kawamoto et al., 2007). In this study, a programmable freeze controller was used due to its capability to control the cooling rate. The highest post-thaw sperm motility was achieved at -5 °C/min, which was faster than that used in European flat oyster *Ostrea edulis* (-3 °C/min; Vitiello et al., 2011), but was slower than in other species, such as, disc abalone *H. discus hannai* (-50 °C/min; Kang et al., 2004), small abalone (-12 or -15 °C/min; Gwo et al., 2002), red abalone *H. rufescens* (-16 °C/min; Salinas-Flores et al., 2005), Pacific oysters (-6 °C/min; Ieropoli et al., 2004), scallops (*C. farreri*) (-20 °C/min; Li et al., 2000) and macha surf clams (-18 °C/min; Dupré and Guerrero, 2011).

Thawing temperature is another important factor to the success of sperm cryopreservation as optimal thawing temperature should inhibit recrystallization and also prevent osmotic stress on sperm (Gao and Critser, 2000; Herráez et al., 2012). Among the thawing temperatures evaluated in this study, 40 °C achieved the highest

post-thaw sperm motility. This temperature agreed with that used for Pacific and pearl oysters (Dong et al., 2005b, c, 2006, 2007). However, it was lower than those found by other studies of this species (Liu et al., 2014b), farmed blacklip abalone (Liu et al., in press b), red abalone (Salinas-Flores et al., 2005) and small abalone (Gwo et al., 2002). The higher optimal thawing temperature revealed in the study by Liu et al. (2014b) may be due to a different freezing technique used.

Theoretically, sperm from different species should have their own optimal endpoint temperature and lower or higher temperatures than this will affect sperm cryosurvival (Mazur, 1965; Gwo, 2008). In this study, the highest post-thaw sperm motility was achieved at -30 °C, which was agreed with our recent result in the blue mussels *Mytilus galloprovincialis* (unpublished data). However, this temperature was much higher than that in small abalone (-90 °C) (Gwo et al., 2002). The reasons causing no post-thaw sperm motility at -20 °C endpoint temperature in this study were not clear although it might be due to that the sperm were not dehydrated enough at -20 °C, causing severe damage by intracellular ice formation after being put into LN. This phenomenon has also been observed in marine shrimp *Sicyonia ingentis* (Anchordoguy et al., 1988) and African catfish *Clarias gariepinus* (Viveiros et al., 2000, 2001) where sperm viabilities were also lower at higher endpoint temperatures.

Sugar is usually included as a cryoprotective medium for livestock and fish species to (1) reduce the formation of intracellular ice; (2) stabilize the membrane during freezing; and (3) be utilized as an energy source (Suquet et al., 2000; Vishwanath and Shannon, 2000; Purdy, 2006; Cabrita et al., 2010). Moreover, the increase in the sperm to egg ratio to compensate the damaged sperm is beneficial to increase the post-thaw sperm fertilization rate and has been used in marine mollusc (Tsai and Chao, 1994; Gwo et al., 2002; Dong et al., 2005b, c, 2006, 2007; Salinas-Flores et al.,

2005). In this study, no positive effect on post-thaw motility was revealed by the addition of trehalose and glucose. The addition of 4% sucrose improved the post-thaw sperm motility, but did not improve the post-thaw sperm fertilization rate in the present study. These results were in the agreement with the findings for Japanese pearl oysters *P. fucata martensii* where the post-thaw sperm quality could not be improved by the addition of glucose, sucrose or trehalose (Kawamoto et al., 2007). In contrast, Liu et al. (2014b) found that the addition of 1% glucose could significantly improve the post-thaw sperm fertilization rate in farmed greenlip abalone. The discrepancy between Liu's et al (2014b) and the present study may be due to different freezing techniques used. In their study the liquid nitrogen vapour method was applied.

The addition of amino acids or vitamins in the cryoprotective medium has been used to improve the post-thaw sperm quality in fish and livestock species as they could (1) reduce oxidative stress and (2) inhibit lipid peroxidation (He and Woods, 2003, 2004; Li et al., 2003; Purdy, 2006; Cabrita et al., 2011; Martínez-Páramo et al., 2012, 2013). In this study, post-thaw sperm motility was significantly improved by the addition of glycine, taurine or L-ascorbic acid, whereas the post-thaw sperm fertilization rate was only improved by addition of 0.6% glycine in 10% DMSO. This result was similar to the findings in Pacific and American oysters *C. virginica* where 0.6% glycine was preferentially selected (Zell et al., 1979; Yankson and Moyse, 1991; Zhang et al., 2012). Glycine improvement in post-thaw sperm quality has also been reported in other non-molluscan species, such as Cynomolgus monkey *Macaca fascicularis* (Li et al., 2003), buffalo bull (El-Sheshtawy et al., 2008), striped bass *Morone saxatilis* (He and Woods, 2004) and Moghani ram (Khalili et al., 2010). This enhancement could be explained by the protective effects of glycine on post-thaw

sperm mitochondrial membrane potential and acrosome integrity that were revealed in the present study.

5.6 Conclusions

The current study has demonstrated that among the three permeable CPAs evaluated 10% DMSO was optimal for the cryopreservation of greenlip abalone sperm using programmable freezing technique. The addition of 0.6% glycine in 10% DMSO further improved the post-thaw fertilization rate to the level similar to the control although the sperm to egg ratios required were higher than in the control.

CHAPTER 6 : IMPROVEMENT IN NON-PROGRAMMABLE SPERM CRYOPRESERVATION TECHNIQUES IN FARMED GREENLIP ABALONE *HALIOTIS LAEVIGATA*[*]

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6.1 Abstract

This study assessed the effects of the addition of vitamin (L-ascorbic acid), amino acids (glycine and taurine), and monosaccharides (glucose, fructose and galactose) on sperm cryopreservation using a non-programmable freezing technique in farmed greenlip abalone. The results showed that the addition of taurine, glycine or L-ascorbic acid significantly improved the post-thaw sperm motility, whereas the post-thaw sperm fertilization rates were improved by the addition of glycine or L-ascorbic acid. Flow cytometry analysis demonstrated that the addition of glycine significantly enhanced the post-thaw sperm plasma membrane integrity and acrosome integrity. Results from the investigation on monosaccharides demonstrated that glucose, fructose and galactose had a similar cryoprotective effect, resulting in a similar level of the post-thaw sperm fertilization rate, plasma membrane integrity, mitochondrial membrane potential and acrosome integrity. In this study, the highest post-thaw fertilization rate of 96% was achieved by using the cryoprotective mediums containing 6% DMSO, 1% glucose and 0.6% glycine.

Keywords: farmed greenlip abalone, non-programmable freezing technique, vitamin, amino acid, monosaccharides

6.2 Introduction

In Australia, genetic improvement programs, such as selective breeding and hybridization, have been established to improve the production and maintain the sustainable and competitive long-term development of the abalone aquaculture industry (Li, 2008). Recently, cryopreservation techniques have been developed and have potential for enhancing the efficiency of these programs by overcoming the asynchronous spawning between male and female in greenlip abalone *Haliotis laevigata* on farm (Liu et al., 2014b). In comparison with sperm collected from wild greenlip abalone (Zhu et al., 2014), those collected from farmed broodstock were more sensitive to cryopreservation. To achieve a post-thaw sperm fertilization rate of about 90% in farmed stocks, a high sperm to egg ratio of 40000:1 and 10000:1 was required in programmable and non-programmable freezing techniques, respectively (Liu et al., in press a; Liu et al., 2014b). For successful application of this technique in commercial hatchery production or genetic improvement programs, a higher fertilization rate is needed using a lower sperm to egg ratio. One of the aims of this study is to investigate whether higher fertilization rates using a lower sperm to egg ratio can be achieved with the addition of amino acids and vitamins when using the non-programmable freezing technique.

Sugars have been widely used as a part of sperm cryoprotective mediums in livestock and fish species. Sugars are thought to lead to more favourable osmotic pressures for the purpose of inducing sperm dehydration and reducing the incidence of intracellular ice formation, resulting in the maintenance of sperm quality during cryopreservation (Horváth et al., 2003; Gómez-Fernández et al., 2012). Glucose has been shown to play a positive role in sperm cryopreservation using the non-

programmable freezing technique in farmed greenlip abalone (Liu et al., 2014b). In other studies, fructose and galactose can provide better cryoprotection than glucose in canine (Ponglowhapan et al., 2004), dog (Yildiz et al., 2000) and red deer (Fernández-Santos et al., 2007). The effects of different types of monosaccharides on sperm cryopreservation have not been investigated in abalone and other marine mollusc species. Therefore, this study will investigate the effects of sugars, amino acids and vitamins on the success of cryopreservation for farmed greenlip abalone which may be applicable to other marine mollusc.

6.3 Materials and methods

6.3.1 Animals and gamete preparation

Three year old farmed animals were provided by the SAM Abalone, Port Lincoln, South Australia (SA) in early October and were transported by air to the Aquatic Sciences Centre, South Australian Research and Development Institute (SARDI), Adelaide, SA. Methods for animal acclimation, spawning induction, and collection of concentrated sperm were described by Liu et al. (2014b). Eggs were gently poured through a 300 μm sieve and retained on a 90 μm sieve at the bottom. The collected eggs were gently rinsed with 5 μm filtered seawater and then washed into a settlement beaker. After 15 min the eggs on the bottom were transferred into another container and adjusted to a density of 1×10^4 mL^{-1}. The eggs were used in the subsequent experiments in 2 h post spawning.

6.3.2 Chemicals

Dimethyl sulfoxide (DMSO), glucose, fructose, galactose, glycine, taurine and L-ascorbic acid were in AR grade and purchased from Sigma-Aldrich Pty Ltd. Their

stock solutions were prepared using 5 μm filtered seawater at double the concentration required in experiments. When the stock solution was mixed with sperm at a 1:1 ratio (v/v), the final concentration was reached.

The LIVE/DEAD sperm viability kit (L-7011) for plasma membrane integrity (PMI) evaluation and LysoTracker green DND-26 (LYSO-G) kit (L-7526) for acrosome integrity (AI) evaluation were purchased from Invitrogen Australia. Rhodamine 123 (Rh 123) for mitochondrial membrane potential (MMP) evaluation and propidium iodide (PI) used for AI and MMP evaluation were purchased from Sigma-Aldrich Pty Ltd. The working solutions of these fluorescent agents were prepared according to Liu et al. (2014b).

6.3.3 Sperm cryopreservation and quality evaluations

The sperm cryopreservation protocol optimized by Liu et al. (2014b) was applied in this study. Briefly, the pre-cold concentrated sperm were mixed directly with the pre-cold cryoprotective medium at a 1:1 ratio and stored on ice for 10 min. The mixture was then transferred into 0.25 mL straws and placed on a rack 5.2 cm above the surface of liquid nitrogen (LN) in a styrofoam box. The straws were exposed to LN vapour for 10 min before being stored in LN for at least 24 h. The straws were thawed in 60 °C and recovered in 18 °C seawater baths.

The motility rate was determined by diluting the sperm suspension 5 times with 5 μm filtered seawater and then counting the number of active sperm out of 100 by two independent observers. Sperm moving forward progressively were counted as active sperm while those vibrating or not moving at all were counted as dead sperm.

The fertilization assessment was conducted in 10 mL tubes. During fertilization, the required volume of concentrated eggs was taken using a pipette and mixed gently

with post-thaw sperm to reach the specific sperm to egg ratio required in experiments. After contact for 10 min, the eggs were washed gently on a 90 μm sieve by 5 μm filtered seawater prior to being cultured in a 500 mL container. The fertilization rate was determined microscopically 4 h post-fertilization by counting the number of fertilized eggs out of a sample of 100. Eggs with two or multiple cells were counted as fertilized eggs and the rest was counted as unfertilized eggs.

For PMI, MMP and AI evaluations, thawed sperm were diluted to 2×10^6 sperm/mL and 1 mL of diluted sperm was then stained fluorescently prior to flow cytometric analysis. For PMI and MMP assessments, 100 μL SYBR14 or Rh123 was added for 20 min, respectively, and then 100 μL PI for a further 10 min. For AI analysis, 5 μL LYSO-G was added for 30 min, and then 9 μL PI for a further10 min. All staining was carried out at the room temperature. The BD FACSVerse flow cytometer was used with 488 nm argon laser. The FACSuite software provided by the manufacturer was applied to calibrate the instrument settings. A total of 10000 events were read in each sample. Density plots were generated with green fluorescence for sperm with intact plasma membrane (SYBR 14), functional mitochondria (Rh 123) or intact acrosome (LYSO-G) and red fluorescence for dead sperm (PI). Their percentages were determined using Flowing Software (University of Turku, Finland).

6.3.4 Experiments

6.3.4.1 Effects of different monosaccharides on post-thaw sperm quality in farmed greenlip abalone

In this experiment, the effects of the addition of 1% glucose, fructose or galactose in 6% DMSO on post-thaw sperm PMI, MMP, AI and fertilization rate, at sperm to egg ratios of 2000:1, 6000:1 and 10000:1, were assessed. The addition of 0.6% glycine

(optimized from Experiment *6.3.4.2*) to each of the monosaccharides was further evaluated using the same quality indicators at sperm to egg ratios of 500:1, 1000:1 and 2000:1. This and subsequent experiments were repeated three times using different sperm pools.

6.3.4.2 Effects of the addition of amino acid and vitamin on post-thaw sperm quality in farmed greenlip abalone

In this experiment, effects of the addition of glycine (0.4, 0.6, 0.8 or 1.0%), taurine (0.05, 0.1, 0.2 or 0.4%) or L-ascorbic acid (0.01, 0.02 or 0.04%) to the cryoprotective medium (6% DMSO + 1% glucose) optimized by Liu et al. (2014b) on post-thaw sperm motility were evaluated. Post-thaw sperm fertilization rates with 6% DMSO + 1% glucose, combined with either the optimal glycine (0.6%), taurine (0.4%) or L-ascorbic acid (0.02%), at sperm to egg ratios of 2000:1, 6000:1 and 10000:1, were compared. The PMI, MMP and AI evaluations were conducted using sperm cryopreserved in 6% DMSO + 1% glucose (control) and combined with 0.6% glycine or 0.02% L-ascorbic acid.

6.3.5 Statistical analysis

Results in this study were presented as mean ± standard deviation (SD). The original data were arcsine transformed before the analysis with SPSS 20. One-way analysis of variance (ANOVA) was applied to analyse the effects of cryoprotective mediums on post-thaw sperm motility, PMI, MMP and AI. Two-way ANOVA was applied in the experiment to assess the effects of cryoprotective mediums and sperm to egg ratios on post-thaw sperm fertilization rate. The Least-Significant Difference (LSD) comparison was used when a significant difference was observed at $P < 0.05$.

6.4 Results

6.4.1 Effects of different monosaccharides on post-thaw sperm quality in farmed greenlip abalone

No significant difference in post-thaw sperm fertilization rates was found when sperm was cryopreserved in 6% DMSO plus 1% glucose, galactose or fructose at sperm to egg ratios of 2000:1 and 6000:1 ($P > 0.05$) (Fig. 6. 1). Although 6% DMSO + 1% glucose produced significantly higher post-thaw sperm fertilization rates than the other two monosaccharides at a sperm to egg ratio of 10000:1 ($P < 0.05$), the addition of galactose or fructose also resulted in high fertiliation rates of 93% (Fig. 6.1). Evaluation of PMI, MMP and AI showed that the differences in each quality assessment indicator between the three cryoprotective mediums were not significant ($P > 0.05$) (Table 6.1). When 0.6% glycine was added, the sperm to egg ratio required to achieve about 90% fertilization rate was reduced to 2000:1 in all the treatments (Table 6.2). At the sperm to egg ratio of 1000:1, the cryoprotective medium containing 1% glucose produced a significantly higher post-thaw sperm fertilization rate than the other two monosaccharides ($P < 0.01$ in fructose, and $P < 0.05$ in galactose) (Table 6.2). In addition, difference in post-thaw sperm PMI, MMP and AI was not significant between three monosaccharides after the addition of 0.6% glycine in the cryoprotective mediums ($P > 0.05$) (Table 6.3).

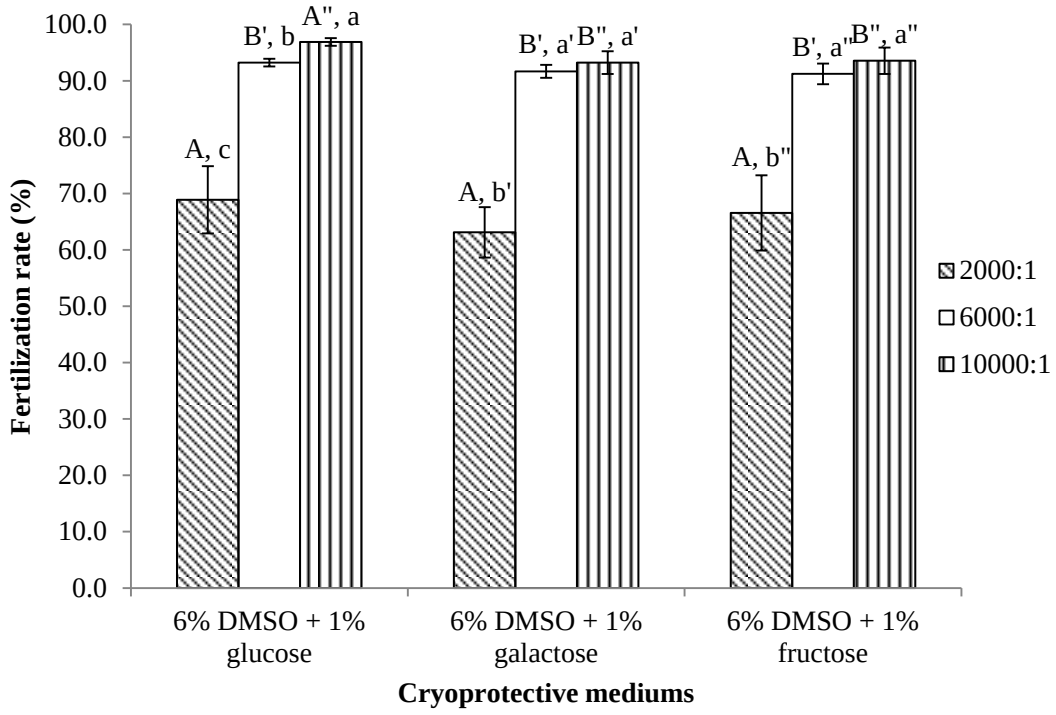

Figure 6-1 Comparisons of post-thaw sperm fertilization rate (%) at different sperm to egg ratios in sperm cryopreserved in 6% DMSO plus different monosaccharides in farmed greenlip abalone, $n = 3$. Bars with different capital letters in each sperm to egg ratio indicate significant difference ($P < 0.05$) between cryoprotective mediums. Bars with different lowercase letters in each cryoprotective medium indicate significant difference ($P < 0.05$) between different sperm to egg ratios.

Table 6.1 Comparison of plasma membrane integrity, mitochondrial membrane potential and acrosome integrity between sperm cryopreserved in 6% DMSO plus different monosaccharides in farmed greenlip abalone, $n = 3$.

Cryoprotective mediums	Sperm component and organelle		
	PMI (%)	MMP (%)	AI (%)
6% DMSO + 1% glucose	39.3 ± 5.8%[a]	43.6 ± 7.4%[a]	47.7 ± 0.6%[a]
6% DMSO + 1% fructose	36.9 ± 5.5%[a]	44.7 ± 1.1%[a]	43.8 ± 5.8%[a]
6% DMSO + 1% galactose	37.1 ± 3.8%[a]	38.7 ± 4.4%[a]	46.4 ± 1.4%[a]

Same letter within each assessment parameter indicates the difference is not significant ($P > 0.05$).

Table 6.2 Post-thaw sperm fertilization rates (%) between sperm cryopreserved in selected cryoprotective mediums in farmed greenlip abalone, $n = 3$.

Cryoprotective mediums	Sperm to egg ratios		
	500:1	1000:1	2000:1
6% DMSO + 0.6% glycine + 1% glucose	68.2 ± 7.0%$^{A, c}$	81.1 ± 6.2%$^{A', b}$	93.6 ± 1.6%$^{A'', a}$
6% DMSO + 0.6% glycine + 1% galactose	61.2 ± 8.8%$^{AB, b'}$	63.3 ± 10.8%$^{B', b'}$	91.7 ± 1.2%$^{A'', a'}$
6% DMSO + 0.6% glycine + 1% fructose	50.6 ± 7.5%$^{B, b''}$	51.7 ± 3.8%$^{C',b''}$	86.8 ± 3.1%$^{A'', a''}$

Different capital letters in each sperm to egg ratio indicate significant difference ($P < 0.05$) between cryoprotective mediums. Bars with different lowercase letters in each cryoprotective medium indicate significant difference ($P < 0.05$) between different sperm to egg ratios.

Table 6.3 Comparison of plasma membrane integrity, mitochondrial membrane potential and acrosome integrity between sperm cryopreserved in 6% DMSO + 0.6% glycine plus different monosaccharides in farmed greenlip abalone, $n = 3$.

Cryoprotective mediums	Sperm component and organelle		
	PMI	MMP	AI
6% DMSO + 0.6% glycine + 1% glucose	54.0 ± 5.1%^{a}	50.8 ± 3.0%^{a}	57.0 ± 1.5%^{a}
6% DMSO + 0.6% glycine + 1% fructose	48.6 ± 2.2%^{a}	48.5 ± 5.0%^{a}	49.7 ± 6.1%^{a}
6% DMSO + 0.6% glycine + 1% galactose	52.2 ± 4.7%^{a}	46.8 ± 2.9%^{a}	51.4 ± 6.8%^{a}

Same letters within each assessment parameter indicate difference is not significant ($P > 0.05$).

6.4.2 Effects of the addition of amino acid and vitamin on post-thaw sperm quality in farmed greenlip abalone

Post-thaw sperm motility was significantly improved by the addition of taurine ($P < 0.05$) (Fig. 6.2), 0.6 and 0.8% glycine ($P < 0.01$ and 0.05, respectively) (Fig. 6.3) and 0.02 and 0.04% L-ascorbic acid ($P < 0.05$) (Fig. 6.4). Further comparison of post-thaw sperm fertilization rates between the taurine, glycine and L-ascorbic acid treatments producing the highest post-thaw sperm motility showed that the addition of 0.4% taurine did not improve the post-thaw sperm fertilizations at each respective sperm to egg ratio evaluated. In contrast, the addition of 0.6% glycine or 0.02% L-ascorbic acid significantly improved the rate at the sperm to egg ratio of 2000:1 ($P <$

0.01) with the highest rate of 96% being produced in the former (Fig. 6.5). When the ratio increased to 6000:1, only the addition of 0.6% glycine significantly enhanced the post-thaw sperm fertilization rate ($P < 0.05$). No improvement was produced when the ratio increased to 10,000:1. Flow cytometry evaluation demonstrated that the addition of 0.6% glycine and 0.02% L-ascorbic acid significantly improved the post-thaw sperm PMI ($P < 0.05$) and AI ($P < 0.01$), and PMI, MMP and AI ($P < 0.05$), respectively (Table 6.4).

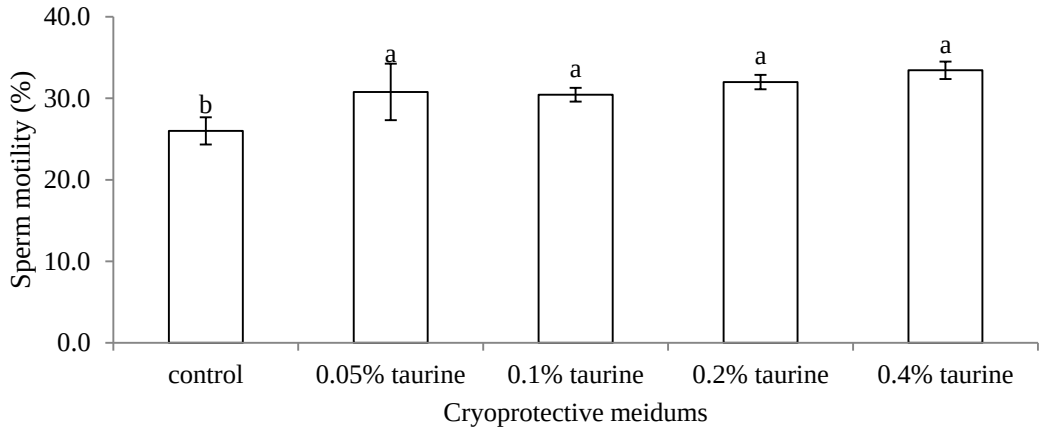

Figure 6-2 Comparisons of post-thaw sperm motilities (%) between sperm cryopreserved in 6% DMSO + 1% glucose (control) and combinations of 6% DSMO + 1% glucose with different concentrations of taurine in farmed greenlip abalone, $n = 3$. Bars with different letters indicate significant difference ($P < 0.05$).

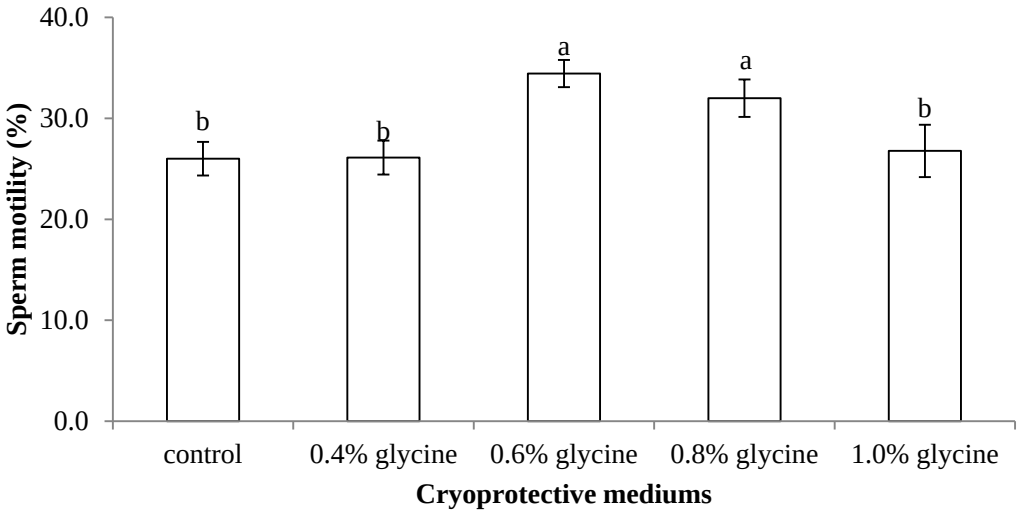

Figure 6-3 Comparisons of post-thaw sperm motilities (%) between sperm cryopreserved in 6% DMSO + 1% glucose (control) and combinations of 6% DSMO + 1% glucose with different concentrations of glycine in farmed greenlip abalone, *n* = 3. Bars with different letters indicate significant difference ($P < 0.05$).

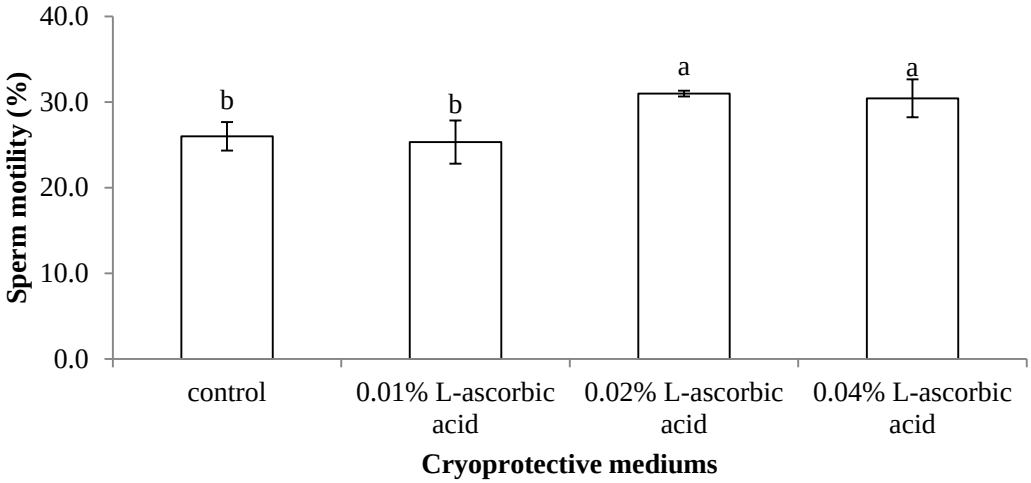

Figure 6-4 Comparisons of post-thaw sperm motilities (%) between sperm cryopreserved in 6% DMSO + 1% glucose (control) and combinations of 6% DSMO + 1% glucose with different concentrations of L-ascorbic acid in farmed greenlip abalone, *n* = 3. Bars with different letters indicate significant difference ($P < 0.05$).

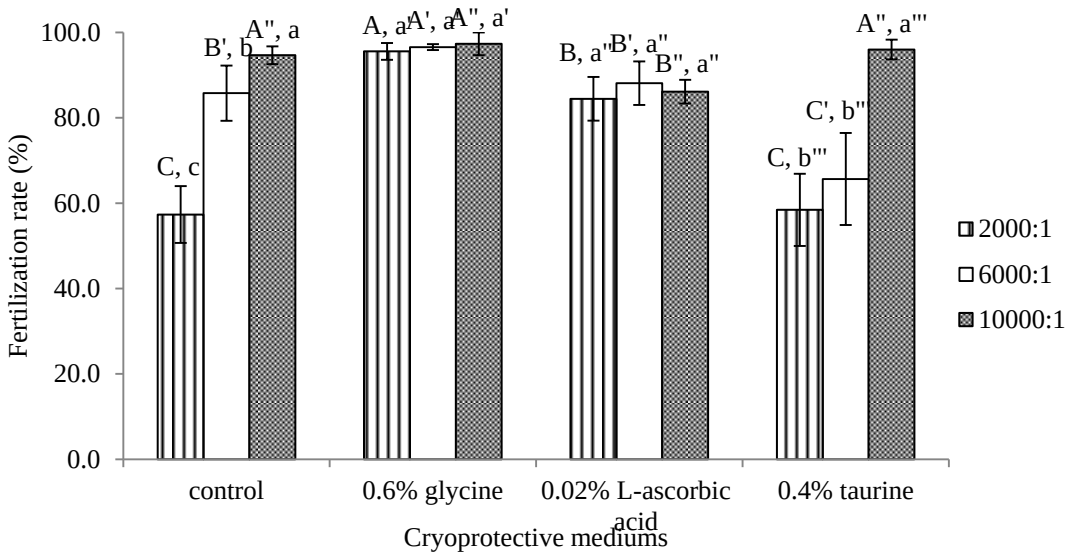

Figure 6-5 Comparisons of post-thaw sperm fertilization rate (%) at different sperm to egg ratios between sperm cryopreserved in 6% DMSO + 1% glucose (control) and combinations of 6% DMSO + 1% glucose with 0.6% glycine, 0.02% L-ascorbic acid or 0.4% taurine in farmed greenlip abalone, $n = 3$. Different capital letters in each sperm to egg ratio indicate significant difference ($P < 0.05$) between cryoprotective mediums. Bars with lowercase letters in each cryoprotective medium indicate significant difference ($P < 0.05$) between different sperm to egg ratios.

Table 6.4 Comparison of plasma membrane integrity, mitochondrial membrane potential and acrosome integrity between sperm cryopreserved in 6% DMSO + 1% glucose and its combination with 0.6% glycine or 0.02% L-ascorbic acid in farmed greenlip abalone, $n = 3$.

Cryoprotective mediums	Sperm component and organelle		
	PMI (%)	MMP (%)	AI (%)
6% DMSO + 1% glucose	24.2 ± 3.3^{c}	39.1 ± 1.1^{b}	33.5 ± 1.4^{c}
6% DMSO + 1% glucose + 0.02% L-ascorbic acid	30.8 ± 2.1^{b}	46.8 ± 3.4^{a}	39.0 ± 1.8^{b}
6% DMSO + 1% glucose + 0.6% glycine	38.2 ± 1.6^{a}	44.3 ± 2.7^{ab}	49.8 ± 4.2^{a}

Different letters within each quality parameter indicate significant difference ($P < 0.05$).

6.5 DISCUSSION

This study has found that the non-programmable sperm cryopreservation technique in farmed greenlip abalone can be improved by the addition of 0.6% glycine in 6% DMSO + 1% glucose. In addition, when glucose, galactose and fructose were applied at the same final concentration, these three monosaccharides showed a similar cryoprotective effect in farmed greenlip abalone.

The supplementation of amino acids and vitamins in cryoprotective medium can improve the post-thaw sperm quality in livestock and fish species by reducing oxidative stress and inhibiting lipid peroxidation (He and Woods, 2004; Li et al., 2003; Martínez-Páramo et al., 2012). In this study, the post-thaw sperm motility increased significantly by the addition of glycine, taurine or L-ascorbic acid at certain concentrations. These results agree with the findings in Moghani ram (Khalili et al., 2010), cynomolgus monkey *Macaca fascicularis* (Li et al., 2003), European sea bass *Dicentrarchus labrax* (Martínez-Páramo et al., 2012), and boer goat (Memon et al., 2013). However, the addition of taurine did not improve the post-thaw sperm fertilization rates at all sperm to egg ratios evaluated in this study. Reasons causing this phenomenon are not known. The addition of glycine or L-ascorbic acid, on the other hand, produced significantly higher post-thaw sperm fertilization rates, and this finding was supported by the improved post-thaw sperm PMI, MMP and AI, and PMI and AI in L-ascorbic acid and glycine treatments, respectively. The addition of glycine yielded the best cryoprotective effect of the glycine and L-ascorbic acid treatments. Previous studies have suggested that glycine can form a layer on the sperm surface by interacting with the phosphate groups within the sperm plasma membrane phospholipids. This layer in turn can

subsequently influence the permeability of DMSO entering into sperm, resulting in the DMSO osmotic or toxic stress being minimized (Anchordoguy et al., 1988; He and Wood, 2003). The benefit of glycine has been also found in *Dama dama* deer (Fernández et al., 2013), and grayling *Thymallus thymallus* (Lahnsteiner et al., 1992).

No significant difference in post-thaw sperm fertilization rate was found with the addition of glucose, galactose or fructose in 6% DMSO, although the addition of glucose resulted in a higher numerical rate than the other two treatments. Flow cytometry results have demonstrated that these three monosaccharides produced similar post-thaw sperm PMI, MMP and AI values, suggesting that sugars with same molecular weight have similar cryoprotective functions (Horváth et al., 2003; Gómez-Fernández et al., 2012). These results agree with the findings in African catfish *Clarias gariepinus* (Urbányi et al., 1999), mouse (Yildiz et al., 2007) and Yorkshire boar (Chanapiwat et al., 2012). However, our results differ from those reported in boar (Gómez-Fernández et al., 2012), canine (Ponglowhapan et al., 2004), dog (Yildiz et al., 2000) and red deer (Fernández-Santos et al., 2007) where fructose or galactose gave better cryoprotection than glucose. These studies showed that the cryoprotective effects of sugars with the same molecular weight might be species specific. Moreover, the addition of 0.6% glycine in cryoprotective mediums containing different monosaccharides further increased the post-thaw sperm fertilization rates, and PMI, MMP and AI between the three monosaccharides evaluated. These results suggest that the positive role of glycine in cryopreservation is not affected by the type of monosaccharide it is mixed with.

In summary, the current study has improved the sperm cryopreservation technique in farmed greenlip abalone, resulting in post-thaw sperm fertilization rates of 96% with the sperm to egg ratio of 2000:1. Moreover, this study has also improved our

understanding on the cryoprotective effects of different monosaccharides in greenlip abalone. The technique has established the foundation toward enhancing the efficiency of genetic improvement programs in abalone aquaculture and sperm cryobanking.

CHAPTER 7 : CRYOPRESERVATION OF SPERM IN FARMED BLACKLIP ABALONE (*HALIOTIS RUBRA*)*

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7.1 Abstract

This study developed a technique of sperm cryopreservation using liquid nitrogen (LN) vapour in farmed blacklip abalone *Haliotis rubra* through evaluating the following five key factors : (1) cryoprotectant agent (CPA) toxicity; (2) cooling temperature; (3) thawing temperature; (4) sperm to egg ratio; and (5) sugar addition, using sperm motility or fertilization rate as quality assessment indicators. The results demonstrated that 6% dimethyl sulfoxide (DMSO) was the best single CPA for sperm cryopreservation in this species. The highest post-thaw sperm motility was achieved when sperm were exposed to LN vapour for 10 min at 5.2 cm above the LN surface and thawed at 60ºC and recovered at 16ºC in seawater baths. Post-thaw sperm motility was found to be significantly higher when 6% DMSO was used in combination with 1% or 2% glucose than 6% DMSO alone. Further evaluation of fertilization rate between these CPAs showed that 6% DMSO + 2% glucose achieved the highest fertilization rate of 70% at a sperm to egg ratio of 10000:1.

Keywords: farmed blacklip abalone, *Haliotis rubra*, sperm cryopreservation, glucose

7.2 Introduction

The blacklip abalone *Haliotis rubra* is one of the most important abalone species in the world (Goodsell et al., 2006), and also critical to some states in Australia, such as New South Wales, where the abalone fishery is entirely dependent on this species (Heasman et al., 2007). However, the wild population has substantially declined due to overfishing in some regions (Goodsell et al., 2006). At the same time, abalone aquaculture has developed and expanded quickly in Australia over the last decade (Goodsell et al., 2006). To further improve the productivity and the long-term sustainability of the industry, genetic improvement techniques, such as triploidy (Liu et al., 2004a, b), selective breeding (Li, 2008) and hybridization (Hamilton et al., 2009) have been evaluated and the latter two have been applied. Risks and constraints on the genetic improvement of abalone are caused by the inability to mate particular superior broodstock combinations due to asynchronous spawning and an inability to "back up" the latest generation of genetically improved stock in isolation from the natural environment. For example, all farms currently rely on the intake of sea water from the natural environment, and the occurrence of abalone viral ganglioneuritus in Victoria in 2006 destroyed the entire breeding nucleus which was housed on two farms at that time.

Sperm cryopreservation technique has been applied to address similar issues in livestock and has become a near-billion-dollar business globally (Purdy, 2006; Tiersch, 2008; Mocé and Vicente, 2009). For instance, use of cryopreserved sperm is being routinely applied in equine reproduction and in bull breeding programs (Janett et al., 2003; Bhakat et al., 2011). In aquatic species, this technique has been largely investigated in fish species (Suquet et al., 2000; Muchlisin, 2005; Cabrita et al.,

2010). Research in marine mollusc has increased over the last decade and the sperm cryopreservation has been successfully achieved in some species, such as oysters (Dong et al., 2005b, c, 2006, 2007a, b; Adams et al., 2008). In the Pacific oyster *Crassostrea gigas*, this technique has been of assistance to selective breeding and triploidy genetic improvement programs (Dong et al., 2005b; Adams et al., 2008). It is anticipated that the application of this technique in farmed blacklip abalone could provide similar benefits to the abalone aquaculture industry.

Sugar supplementation is a common strategy to improve the post-thaw sperm quality in livestock, such as pig, goats and sheep, as it enhances the sperm's resistance against cryodamage by increasing the membrane fluidity and reducing intracellular ice formation (Barbas and Mascarenhas, 2009; Gómez-Fernández et al., 2012). Sugars can also be utilized as an energy source in terms of glycolysis to support sperm motility and movement (Purdy, 2006). In fish species, sugar is usually included as a part of the cryoprotective medium (Muchlisin, 2005; Cabrita et al., 2010). The application of sugar in marine mollusc has been found to be species specific. The post-thaw sperm quality was improved by the addition of trehalose in blacklip pearl oysters *Pinctada Marganitifera* (Lyons et al., 2005) and glucose in farmed greenlip abalone *H. laevigata* (Liu et al., 2014b). However, the use of glucose and trehalose is not effective for sperm cryopreservation in small abalone *H. diversicolor* (Tsai and Chao, 1994) and the Japanese pearl oysters *P. fucata martensii* (Kawamoto et al., 2007). So far, no information of sperm cryopreservation on farmed blacklip abalone is available. In this study, factors important to the development of this technique in this species as well as the effects of sugars on the improvement of post-thaw sperm quality were evaluated.

7.3 Materials and methods

7.3.1 Animal sources and gamete collection

The farmed blacklip abalone broodstock used in this study were conditioned at the Great Southern Waters (GSW), Victoria, Australia. The animals were maintained on a flow through system with 10 μm filtered seawater (FS) at 15.0 ºC and fed with an artificial diet (Skretting Australia). The system was cleaned each day before feeding. The broodstock were checked before spawning induction and only animals with large and swollen gonads were selected. A total of twenty-four mature broodstock were selected each time and placed into six glass aquaria (four individuals in each). Spawning was induced by a combination of temperature shock and ultraviolet (UV) irradiation. To collect concentrated sperm the water supply to the aquaria was turned off about 15 ~ 20 min after commencement of spawning and the animals were rinsed in FS to remove the UV irradiated water. The spawning animals were then left undisturbed in the aquaria without further addition of FS to allow them dry spawning and collect sperm using transfer pipette subsequently. The collected sperm were stored in a refrigerator (0 ~ 4 °C) until they were pooled from at least five individuals with the same volume in each male for subsequent experiments within 2 h of spawning. The pooled sperm concentration was determined under a light microscope with a hemacytometer using diluted subsamples. In this study, sperm with initial motility above 85% was used and its concentration was standardized to 1.6×10^8 mL^{-1}.

Twenty females were induced to spawning at each time. Eggs from at least 5 individuals with the same volume were gently siphoned into a sieve set in the bucket with a 300 μm sieve on top to remove large debris and a 90 μm sieve below to retain

the eggs. The collected eggs were gently rinsed by FS and then washed into a settlement beaker. After 15 min, the density of eggs on the bottom was determined to be 1×10^3 mL^{-1}. Eggs used in this study were within 2 h of spawning.

7.3.2 Chemical and equipment preparation

Dimethyl sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG), glycerol, sucrose, glucose and trehalose were AR grade and purchased from Sigma-Aldrich Pty Ltd. The working solutions for each reagent were prepared in fresh FS at double the final concentration needed in each experiment. When the working solution was mixed with sperm at a 1:1(v/v) ratio, the required final concentration was produced.

A styrofoam box (39.0 × 24.5 × 35.5 cm) and the rack with different heights were used in this study. Liquid nitrogen (LN) was added into the styrofoam box to a depth of 2 cm before a rack floating on the LN surface was placed. The lid of styrofoam box was partially closed after 0.25 mL straws (Minitube, Germany) were placed on the rack. Two seawater baths were used to thaw and recover the cryopreserved sperm. The required thawing temperature was achieved by mixing ambient and boiled seawater and monitored by a digital thermometer in a 10 L water bath (Thermo Scan, Eutech Instruments, Singapore). A 16 ºC recovery temperature was achieved by mixing ambient and cold seawater.

7.3.3 Sperm quality assessment

The motility rate was determined by diluting the sperm suspension 5 times with 5 μm FS and then counting the number of active sperm out of 100. The sperm moving forward progressively were counted as active sperm while those vibrating or not moving were counted as dead sperm (Gwo et al., 2002). Each sample was assessed by two independent observers. To standardize across the experiments, the percentage

of fresh sperm motility was set to 100% and the post-thaw sperm motility rate was calculated as a percentage of the fresh sperm motility (Kawamoto et al., 2007).

The required volume of concentrated eggs was taken before being fertilized by the post-thaw sperm at a desired sperm to egg ratio and stirred gently by hand. Ten min later the eggs were washed gently on a 90 μm sieve with FS prior to being reared in a 500 mL container. The FS in the container was changed partially every 30 min for 4 times. The controls were established using a sperm to egg ratio of about 50:1. The fertilization rate was calculated microscopically 4 h post-fertilization by counting the number of fertilized eggs out of a subsample of 100. The eggs with two or multiple cells were counted as fertilized eggs while the rests were counted as unfertilized eggs.

7.3.4 Experiments

In this study, each experiment was repeated 3 times using 3 pools.

7.3.4.1 Effects of CPA types and concentrations on sperm motility

In this experiment, DMSO, EG, PG and glycerol at 4, 8, 12, 16 and 20% concentrations were evaluated. Sperm were mixed with a pre-cold CPA at a 1:1 ratio on ice. After 10 min equilibration, sperm subsamples were collected to assess motility.

7.3.4.2 Effects of equilibration times on sperm motility

The EG, PG and DMSO at a final concentration of 8% were selected in this experiment according to the results from the previous experiment. In this experiment, equilibration times of 10, 20, 30 and 60 min were evaluated. The other procedures were the same as in above section.

7.3.4.3 Effects of rack heights on sperm motility

An equilibration time of 10min was selected in this and subsequent experiments. In this experiment, rack heights of 1.3, 2.6, 3.9, 5.2 and 6.5 cm above the LN surface were evaluated in the styrofoam box. After equilibration on ice, the sperm and CPA mixtures were then transferred into 0.25 mL straws and placed on a rack at the required height. The straws were exposed to LN vapour for 10 min before being put into LN for storage for at least 2 h. The sperm were thawed and recovered in 60 °C and 16 °C seawater baths, respectively before subsamples were collected for motility assessment.

7.3.4.4 Effects of thawing temperatures on sperm motility

In the previous experiment, the sperm frozen at the 5.2 cm height resulted in the highest post-thaw sperm motility. Thus this height was used in this and the subsequent experiments. In this experiment, 40 (7 s), 50 (6 s), 60 (5 s), 70 (4 s) and 80 °C (3 s) thawing temperatures were evaluated using sperm cryopreserved with 6, 8 or 10% DMSO. The other procedures were the same as described in above section.

7.3.4.5 Effects of sugar types and concentrations on sperm motility

The parameters (60 °C thawing temperature and 6% DMSO) achieved the highest post-thaw sperm motility in the previous section were also applied in this experiment to evaluate the effects of addition of 1, 2, 3 or 4% sucrose, glucose and trehalose on post-thaw sperm motility. The other procedures were the same as described in above section.

7.3.4.6 Comparisons of sperm to egg ratios on fertilization rate

Six percent DMSO and its combination with 1 or 2% glucose were used in this experiment to compare the effect of different sperm to egg ratios (5000:1, 10000:1,

15000:1 and 20000:1) on fertilization rates. The other procedures were the same as in above section except that the sperm quality was evaluated by the fertilization rate described.

7.3.5 Statistical analysis

Results in this study were presented as mean ± standard deviation (SD). The original data were arcsine transformed before the two-way analysis of variance (ANOVA) was applied with SPSS 20. The Least-Significant Difference (LSD) comparison was used when a significant difference was observed at $P < 0.05$.

7.4 Results

7.4.1 Effects of CPA types and concentrations on sperm motility

Farmed blacklip abalone sperm were sensitive to the addition of glycerol which resulted in less than 5% motility even at a low concentration of 4% (Fig. 7.1). For other chemicals, sperm showed significantly higher motility with the addition of DMSO than with the addition of EG ($P < 0.05$ in 4 and 16% and $P < 0.01$% in 8 and 12%) and PG ($P < 0.05$% in 4%, $P < 0.01$% in others) in each CPA concentration less than 16%. However, EG and PG at 8% still achieved about 70% sperm motility.

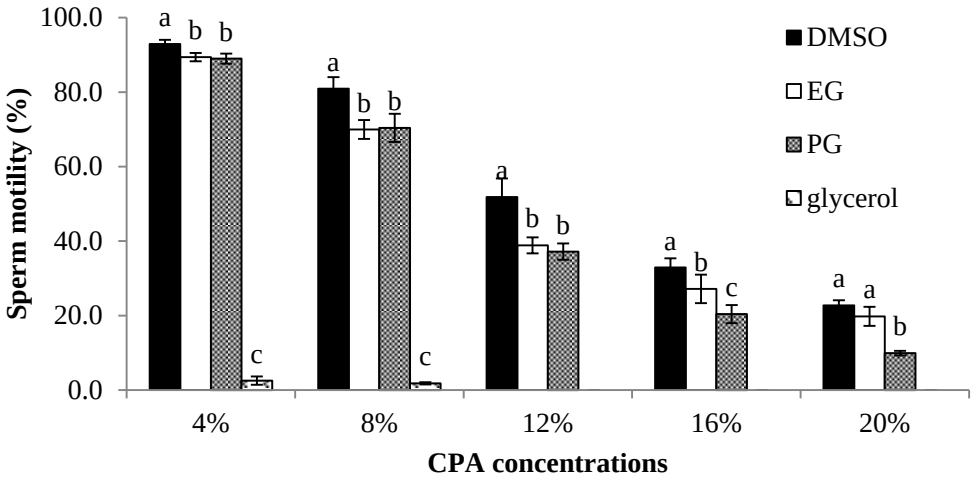

Figure 7-1 Sperm motilities (%) after 10 min exposure to different CPAs at various concentrations on ice, $n = 3$. Different letters within each CPA concentration indicate significant difference between different CPAs.

7.4.2 Effects of equilibration times on sperm motility

The sperm motility was significantly higher in 8% DMSO than in 8% EG or PG at each equilibration time evaluated ($P < 0.05$) (Fig. 7.2). The sperm motility markedly decreased with the expansion of equilibration time from 10 to 60 min ($P < 0.05$),

although this difference was not significant between 30 and 60 min in 8% DMSO ($P > 0.05$).

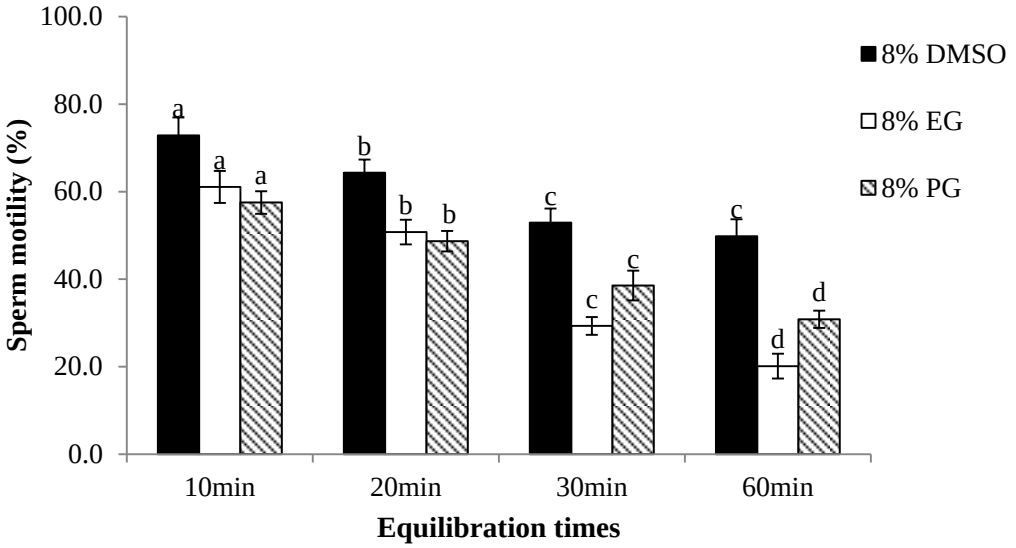

Figure 7-2 Sperm motilities (%) after equilibration in 8% DMSO, EG or PG from 10, 20, 30 or 60 min, $n = 3$. Different letters within each CPA indicate significant difference between different equilibration times.

7.4.3 Effects of rack heights on sperm motility

Post-thaw sperm motility peaked when sperm were frozen at a height of 5.2 cm above the LN surface in each CPA evaluated (Fig. 7.3). At this height, 8% DMSO achieved the highest motility of 30%, which was significantly higher than other CPAs ($P < 0.01$).

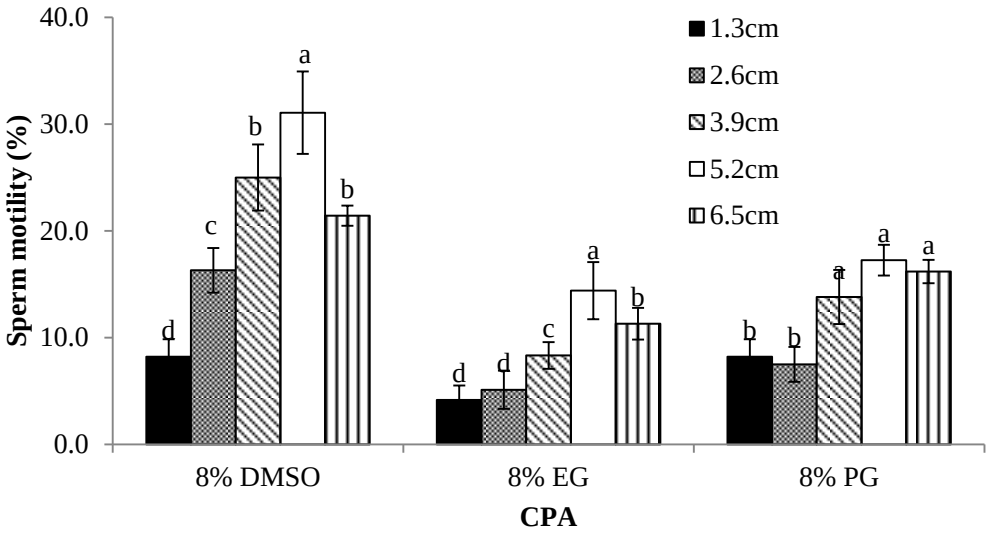

Figure 7-3 Post-thaw sperm motilities (%) after exposure to LN vapour at different rack heights above LN surface in 8% DMSO, EG or PG, *n* = 3. Different letters within each CPA indicate significant difference between different rack heights.

7.4.4 Effects of thawing temperatures on sperm motility

Six percent DMSO produced a higher post-thaw sperm motility than 8 and 10% DMSO at each thawing temperature evaluated (Fig. 7.4). In 6% DMSO, 60°C thawing temperature achieved the highest post-thaw sperm motility (36.7 ± 0.9%), which was significantly higher than other temperatures ($P < 0.05$).

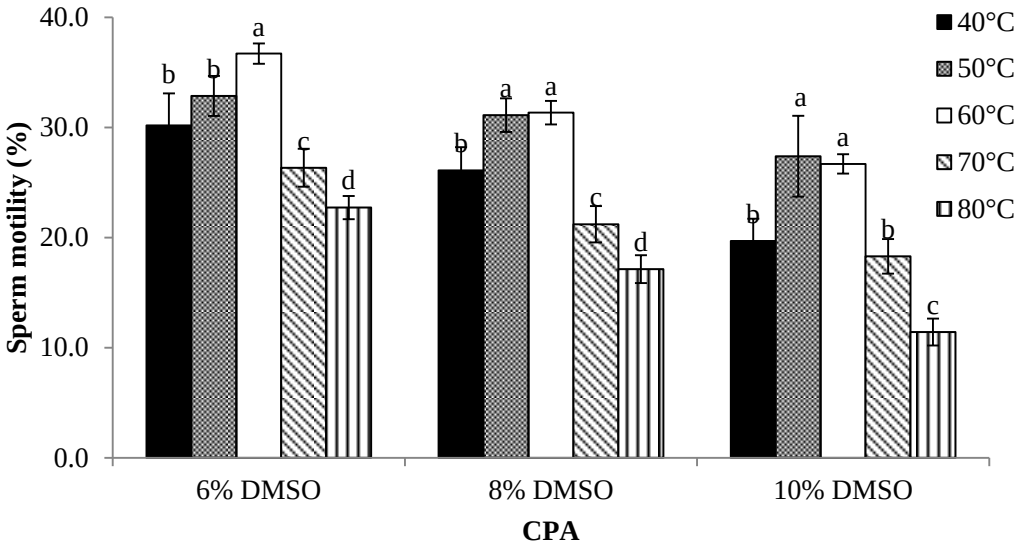

Figure 7-4 Post-thaw sperm motilities (%) after being thawed at different temperatures in 6, 8 or 10% DMSO, n = 3. Different letters within each CPA indicate significant difference between different thawing temperatures.

7.4.5 Effects of sugar types and concentrations on sperm motility

The effects of different types and concentrations of sugars were compared (Fig. 7.5). No significant difference in the post-thaw sperm motility was found among the three controls (6% DMSO) ($P > 0.05$). The addition of 1% or 2% glucose improved the post-thaw sperm motility significantly ($P < 0.001$ and $P < 0.05$, respectively). However, no improvement in the post-thaw sperm motility was revealed by the addition of sucrose or trehalose ($P > 0.05$) (Fig. 7.5).

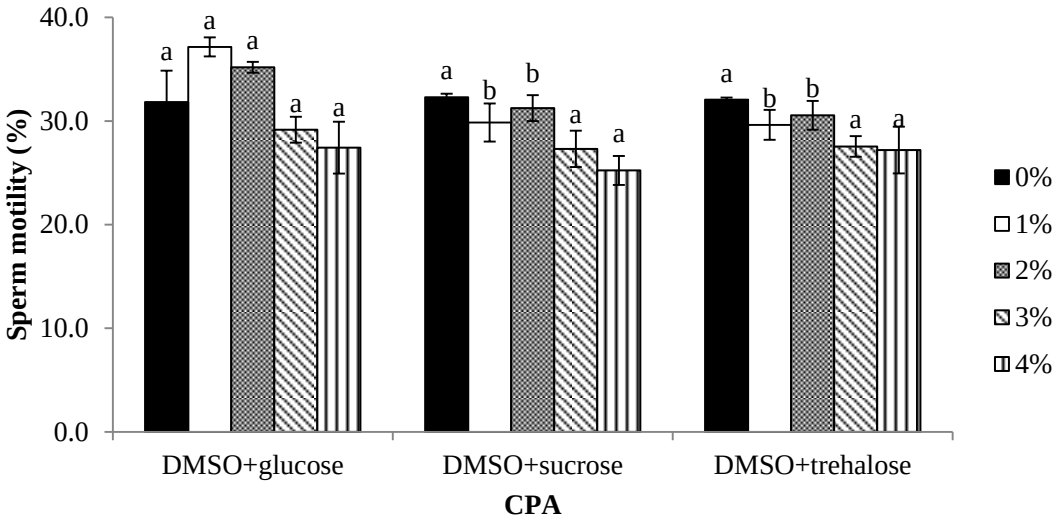

Figure 7-5 Post-thaw sperm motilities (%) after cryopreservation in different types and concentrates of sugars, $n = 3$. Different letters within each concentration indicate significant difference between different CPAs.

7.4.6 Comparisons of sperm to egg ratios on fertilization rate

A sperm to egg ratio of 10000:1 achieved the highest post-thaw sperm fertilization rate (69.0 ± 3.1%) for the sperm cryopreserved with 6% DMSO + 2% glucose, which was significantly higher ($P < 0.05$) than the other treatments. However, this rate was significantly lower than the control (83.0 ± 2.9%) ($P < 0.05$) (Fig. 7.6).

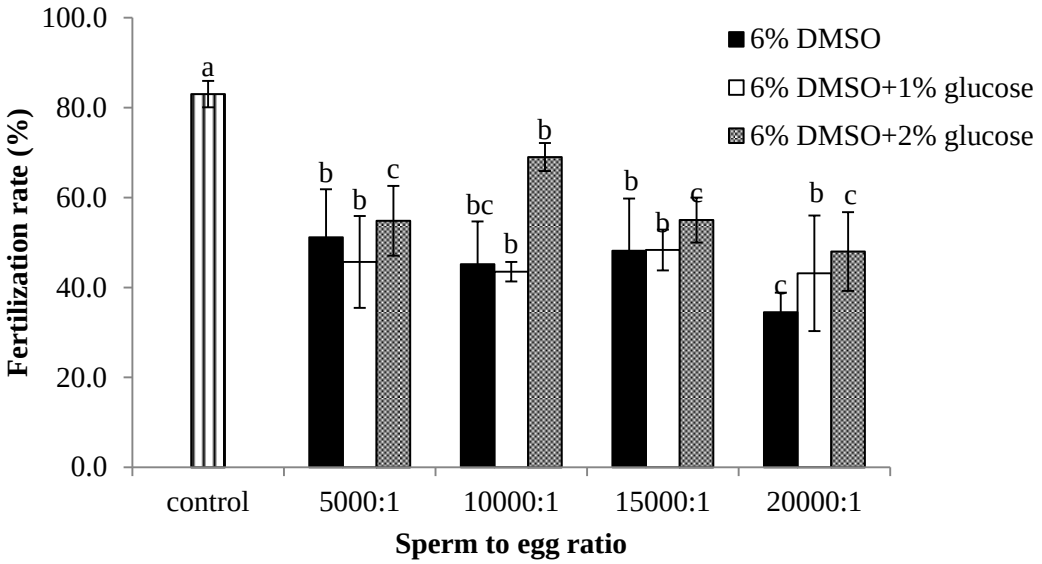

Figure 7-6 Post-thaw sperm fertilization rates (%) at different sperm to egg ratios after cryopreservation in 6% DMSO or its combination with 1% or 2% glucose, $n = 3$. Different letters within each sperm to egg ratio indicate significant difference between different CPAs.

7.5 Discussion

This study has optimized parameters important to the development of the sperm cryopreservation technique in farmed blacklip abalone and achieved about 70% fertilization rate after the addition of 2% glucose in 6% DMSO, suggesting that the addition of glucose may play a positive role in sperm cryopreservation in this species.

CPAs are essential to the development of cryopreservation techniques and their effectiveness is initially determined by the expression of toxic effects during required equilibration times, allowing enough time for a CPA to enter the sperm and protect against cryodamage during cryopreservation (Li, 2005; Sansone et al., 2005; Matteo et al., 2009). Glycerol was found to be the most toxic to farmed blacklip abalone sperm in this study, even at the lowest concentration of 4%. This finding agreed with the results in other abalone species, such as small abalone (Gwo et al., 2002), farmed and wild greenlip abalone (Li, 2005; Liu et al., 2014b) as well as Pacific oysters (Ieropoli et al., 2004; Dong et al., 2005b), Japanese pearl oysters (Kawamoto et al., 2007) and European flat oysters *Ostrea edulis* (Vitiello et al., 2011). On the contrary, glycerol has been shown to be a suitable CPA in giant abalone *H. gigantean* (Matsunaga et al., 1983), disc abalone *H. discus hannai* (Kang et al., 2004) and red abalone *H. rufescens* (Salinas-Flores et al., 2005). These results suggested that the effectiveness of glycerol is species-specific. In other CPAs, a concentration higher than 12% also resulted in a low motility (less than 50%) after incubation for 10 min. This was consistent with the findings in most marine molluscan species, where CPA concentrations between 6 and 12% were frequently used for sperm cryopreservation (Salinas-Flores et al., 2005; Kawamoto et al., 2007; Matteo et al., 2009; Vitiello et al., 2011). Sperm motilities decreased with the increase in equilibration times, although

DMSO decreased slower than EG and PG at the same concentration. Moreover, DMSO showed better cryoprotective effects in comparison with the same concentration of EG and PG. This might be because the permeability of DMSO is less sensitive to low temperature and DMSO can enter the sperm quickly and interact with membrane phospholipids (Suquet et al., 2000; Sansone et al., 2005). In this study, 6% was the best of DMSO concentrations evaluated, which is consistent with the results in wild and farmed greenlip abalone (Li, 2005; Liu et al., 2014b), whereas this concentration was lower than that used in small abalone (Tsai and Chao, 1994; Gwo et al., 2002).

In this study, post-thaw sperm motility was found to be the highest when straws were frozen at 5.2 cm above the LN surface. This height also was the best in farmed greenlip abalone where the temperature at this height was -103°C after 10 min exposure to LN vapour (Liu et al., 2014b). It should be noted that a height higher than this was optimal in Japanese and blacklip pearl oysters (Acosta-Salmón et al., 2007; Kawamoto et al., 2007) and scallops (*Patinopecten yessoensis* and *Chlamys farreri*) (Yang et al., 1999), while a height lower than this was the best in greenshell mussels *Perna canaliculus* (Smith et al., 2012a).

In the current study, a thawing temperature of 60 °C resulted in the highest post-thaw sperm motility. This finding was the same as for farmed greenlip abalone (Liu et al., 2014b), and also similar to other marine mollusc (55 ~ 60 °C), such as Pacific oysters and American oysters *C. virginica* Gemlin (Zell et al., 1979; Yankson and Moyse, 1991). However, this temperature was higher than the optimal temperature for wild greenlip abalone, where 50 °C resulted in the highest post-thaw sperm fertilization rate (Li, 2005).

The addition of sugars into CPA and increase in the sperm to egg ratios have both

been applied as strategies to improve post-thaw sperm quality and fertilization rates, such as blacklip pearl oysters (Lyons et al., 2005), Pacific oysters (Zhang et al., 2012), Eastern oysters (Paniagua-Chávez et al., 2006), red abalone (Salinas-Flores et al., 2005), goat (Purdy, 2006) and fish (Cabrita et al., 2010). In this study, the addition of 1% or 2% glucose in 6% DMSO significantly improved the post-thaw sperm motility, although only the latter markedly improved the post-thaw sperm fertilization rate at a sperm to egg ratio of 10000:1. The reason for fertilization rate improvement may be that the addition of glucose improves the post-thaw sperm plasma membrane integrity and mitochondria membrane potential as found in our previous study in farmed greenlip abalone (Liu et al., 2014b). In farmed greenlip abalone, the addition of 1% glucose achieved the highest fertilization rate, although the sperm to egg ratio was same in both studies (Liu et al., 2014b). This difference in requirement of glucose concentration might be due to differences in sperm characteristics between these two species. The total length of sperm and the sizes of acrosome, nucleus, midpiece and flagellum (length × diameter) were smaller in blacklip abalone than in greenlip abalone (Grubert et al., 2005). Sperm with smaller component sizes may have less lysin and reduced swimming ability, both of which are important factors affecting fertilization (Grubert et al., 2005).

Our study has developed a practical method to cryopreserve the sperm in farmed blacklip abalone, which could be used to improve the efficiency of genetic improvement programs by achieving the desired mating, long-term storage of superior genetics, etc, and off-season commercial seed production as only female broodstock need to be conditioned.

CHAPTER 8 : GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

8.1 General discussion

Genetic improvement programs are desired for the Australian abalone aquaculture industry. However, there are currently several risks that require mitigations and limitations to overcome. First, reliable systems for coordinating the mating of selected individuals are needed as the spawning of individual abalone is difficult to synchronize. Second, a strategy for preservation of superior genetic stock is needed to make it possible to recover the latest selected generations after destruction of the breeding nucleus by natural disasters, including disease outbreaks. Finally, a method is needed for preserving past generations so that cross generational references and evaluations of genetic progress can be made. Sperm cryopreservation is an effective technique for accelerating the efficiency of selective breeding programs, creating safe repositories for preservation of the breeding population and storing reference material that can be used to evaluate genetic progress. In order to develop a suitable sperm cryopreservation protocol for Australian farmed abalone, sperm injuries caused by cryopreservation processes and sperm cryosensitivity generated by broodstock physiological conditions need to be investigated. Understanding these issues is essential for maximizing the biological ability of sperm to produce progeny and will help explain why the sperm of farmed abalone are not well cryopreserved using protocols developed for wild counterparts.

Sperm from farmed greenlip abalone were severely injuried with the non-programmable freezing technique, achieving only 60% post-thaw sperm fertilization rates, even though modifications improving the accuracy of the technique (0.25 mL

straw and floating rack) were applied. This ferlization rate is much lower than achieved for wild counterparts using the same technique (>90%). The significant discrepancy (30%) between fertilization rates indicates that sperm from farmed abalone is more sensitive to the non-programmable freezing technique than that from wild abalone. The reason causing this difference is not clear, but the environmental conditions experienced by wild and farmed animals could be an influence. In abalone aquaculture in Australia, the animals are usually farmed in land-based systems such as shallow concrete slab tanks. The abalone are consistently exposed to relatively higher concentrations of toxic metabolites, such as ammonia and nitrites. These metabolites usaually are not removed as quickly and efficiently as in the wild because of the relatively high animal density and slow water exchange on-farm. In addition, the farmed abalone are fed with artificial diets which may be lower in nutritional quality (Huchette et al., 2003; Grubert et al., 2005), although knowledge about the effects of artificial diets on abalone gonad development are limited. On the contrary, the wild greenlip abalone live on rocky surfaces and steep cliffs at a favourable physico-chemical environment, and feed on natural and high quality food, mainly seaweeds. These differences may explain why the sperm from farmed abalone are much more sensitive to cryopreservation. The first objective has been addressed.

The differences observed in post-thaw sperm fertilization rates between farmed and wild abalone stock indicate room for improvement. Improvement of post-thaw sperm fertilization rates by the addition of certain chemicals have provided the rationale to investigate the response of subcellular structures to different cryotreatments and the cryopretective mechanisms of specific cryoprotectant agents. However, techniques that can differentiate changes at subcellular levels, especially

for sperm components and organelles performing critical roles for abalone fertilization such as sperm plasma membrane, mitochondria and acrosome, are needed. This study has showed that use of fluorescent stains, SYBR 14/propidium iodide (PI), Rhodamine 123/PI and LysoTracker Green DND-26/PI, with fluorescent microscopy and flow cytometry, is an efficient method for revealing sperm plasma membrane integrity (PMI), mitochondrial membrane potential (MMP) and acrosome integrity (AI) in greenlip abalone. Lost integrity of the sperm plasma membrane and acrosome, and decreased functionality of mitochondria, may partially explain why lower post-thaw sperm fertilization rates are achieved in farmed abalone in comparison with wild counterparts. Improvements in post-thaw fertilization rates due to the addition of sugars and/or vitamins and amino acids has been detected, and was found to be associated with either improved PMI, MMP and/or AI. In addition, the efficiency of these evaluations has been substantially enhanced by the employment of flow cytometry, a technique that has been widely used in livestock and finfish sperm cryopreservation studies (Thomas et al., 1997; Cabrita et al., 2010). These results address the third objective.

Sperm collected during the middle of the natural abalone spawning season was found to be less sensitive to cryoinjury and result in higher post-thaw sperm motility, fertilization rate, PMI, MMP and AI values than sperm collected at other times. However, the reasons for achiving higher sperm quality during the middle of a natural spawning season are not clear. Research from finfish species has shown that sperm with high quality are produced when the gonad contains enough proteins and lipids at balanced osmolarity (Lahnsteiner et al., 1998; Papadaki et al., 2008; Rouxel et al., 2008; Bozkurt et al., 2011). In marine molluscs, spermatogenesis needs more lipid and protein from the gonad, and the gonadal development needs a period to

accumulate the nutrient reserves for utilization at a prefered temperatures, photoperiod, etc. (Soudant et al., 1996; Utting and Millican, 1998; Soudant et al., 1999; Martínez et al., 2000; Litaay and Silva, 2003). Litaay and Silva (2003) also found that overall lipid levels in the gonad of blacklip abalone increased up until the peak spawning period. In abalone aquaculture, both 2 and 3 year old animals can be used to produce progeny. However, the use of younger broodstock can enhance genetic progress in a selective breeding program as the generation interval is inversely proportional to the genetic gain. This advantage can be realized by using sperm cryopreservation, as the sperm collected from both age classes show a similar level of cryosensitivity in terms of the post-thaw sperm quality assessment indicators evaluated in this study. These results address the second objective.

It is generally acknowledged that it is impossible to totally avoid sperm injury during cryopreservation. Thus, the development of effective strategies to minimize sperm cryoinjuries is important for sperm cryopreservation. Strategies, such as the application of alternative cryopreservation methods, or the addition of sugars, amino acids and/or vitamins, to minimize the sperm cryoinjuries have been largely investigated and applied in livestock and finfish species to optimize and/or develop sperm cryopreservation protocols. However, these strategies have not been systematically evaluated in marine molluscs. In this study, as the programmable technique did not yield satisfactory results, the non-programmable freezing technique was applied to evaluate the cryoprotective effects of the above-mentioned three categories of chemical. The results showed that abalone sperm cryoinjuries can be mitigated by the addition of any of the three monosaccharides (glucose, galactose and fructose) evaluated. Post-thaw sperm fertilization rate, integrity of sperm components and functionality of organelles were all improved, and this work has

advanced our knowledge about the cryoprotecitve properties of sugars in marine molluscs. Moreover, the cryoinjuries inflicted on sperm are further reduced by optimizing amino acid and vitamin levels. Post-thaw fertilization rates were similar to controls when 0.6% glycine was added to cryoprotective solutions. This is also supported by the improvement of post-thaw sperm PMI, MMP and AI. These results address the fourth and fifth objectives.

When the non-programmable freezing technique is applied in this study, the optimal key cryopreservation parameters were similar between farmed greenlip and blacklip abalone, except that a higher glucose concentrationwas needed in the latter. The reasons for this finding are unknown. However, both species are closely related with a similar biology, and it is possible that both species used in this study were farmed under similar environmental conditions. These results suggest that the procedures used to further improve the sperm cryopreservation protocols for greenlip abalone might also be applicable to blacklip abalone. The last objective has been confirmed.

8.2 Conclusions

This thesis explored the differences in cryo-sensitivities between sperm from farmed and wild abalone and identified the broodstock physiological conditions best suitable for sperm cryopreservation in farmed abalone. Investigation on the methods for sperm quality assessment, sperm component integrity and organelle functionality were investigated. This thesis has resulted in the development of effective strategies for the reduction of sperm cryoinjury in farmed abalone. In summary, the following six conclusions are drawn:

1) Sperm from farmed abalone are more sensitive to cryopreservation treatment than wild counterparts.
2) SYBR 14/PI, Rhodamine 123/PI and LysoTracker Green DND-26/PI are fluorescent dyes that are suitable for the evaluation of sperm plasma membrane integrity, mitochondrial membrane potential and acrosome integrity, respectively. These dyes can be used with either fluorescent microscopy or flow cytometry to assess cryoinjury to sperm components and organelles.
3) In farmed greenlip abalone, post-thaw sperm quality is significantly affected by the timing of sperm collection. Sperm collected during the middle of a natural spawning season (early October in South Australia) is less sensitive to cryoinjury than that collected at other times. Broodstock age (2 or 3 years old), on the other hand, has limited, if any, effect on sperm cryosensitivity.
4) Cryoinjuries on sperm can be reduced with the addition of sugars, vitamins and/or amino acids in farmed greenlip abalone. The three monosaccharides (glucose, galactose and fructose) have similar cryoprotective effects, but give significantly better protection than the disaccharides (sucrose and trehalose) using the non-programmable freezing technique. Sperm cryoinjuries can be

further reduced by the addition of L-ascorbic acid and glycine, with glycine achieving the best post-thaw sperm results.

5) Use of non-programmable freezing technique results in higher post-thaw sperm quality than use of programmable freezing technique and is therefore more suitable for sperm cryopreservation in greenlip abalone.
6) Same optimal values have been revealed in the key cryopreservation parameters evaluated in both farmed greenlip and blacklip abalone when using a non-programmable freezing technique, except that higher glucose concentration is needed to reduce sperm cryoinjury for the latter species.

8.3 Future Research Directions

This PhD research has developed sperm cryopreservation techniques for Australian farmed greenlip and blacklip abalone. In order to integrate this technique into an existing abalone hatchery, other factors should be considered in future studies. Thus, future research should be focussed on the following directions to accelerate the integration of the thesis research outcomes with commercial applications.

1. Increasing the reliabilty and efficiency of broostock husbandry, which is an important requirement for producing high quality sperm as the side-effects caused by inappropriate husbandry have compromised the sperm quality in finfish and livestock species (Fleming et al., 1996; Cabrita et al., 2009; Mocé and Vicente, 2009). Optimization of husbandry conditions can minimize the risks from factors influencing the abalone sperm quality, such as seawater temperature, oxygen, animal density, nutrition and diseases. Moreover, abalone broodstock selection has been based on the gonad observation with lillte attention to the sperm quality. Development of a reliable method to standardize

the broodstock selection would be helpful for the application of sperm cryopreservation technique on commercial farms as the negative broodstock effects can be cumulative and affect the sperm ability to tolerate cryoinjury (Dong, 2005a; Mocé and Vicente, 2009).

2. In abalone, natural spawning is the preferred method for sperm collection and this method produces activated sperm, but sperm quality decreases with the increase of time after collection (Gwo et al., 2002). As high sperm quality is crucial for the success of sperm cryopreservation, it would be beneficial if the sperm can be used in cryopreservation as soon as possible. Furthermore, larger straws or cryo-containers should be evaluated to improve the efficiency of operation. However, it should be noted that the use of larger containers will reduce the ratio between surface area and liquid volume. The difference in surface to volume ratios will influence the velocity of latent heat dissipation, thus affecting the post-thaw sperm quality (Silva et al., 2013; Zhu et al., 2014).
3. The subjective evaluation of motility is a good method to assess sperm quality. Nevertheless, if the computer-assisted sperm analysis system is available, it should be applied to differentiate patterns of the sperm movement as specific sperm moving pattern has been found to be positively correlated with fertilizing ability in some finfish and livestock species (Martinez-Pastor et al., 2005; Flores et al., 2008; Muiño et al., 2009; Ortega-Ferrusola et al., 2009; Beirão et al., 2011), and this might also be true in molluscs.
4. Further efforts should be made to characterise abalone sperm morphology, plasma membrane permeability, fluidity and biochemistry, mitochondria status, acrosomal structures and biochemistry, and DNA integrity, as these variables have been used to as indicators to improve or optimize the sperm

cryopreservation protocols in finfish and livestock speices (Mocé and Graham, 2006; Beirão et al., 2012; Gómez-Fernández et al., 2012; Memon et al., 2013), and may also be applicable to molluscan species.

5. In this study, post-thaw sperm fertilization rates of more than 90% were achieved when freshly produced eggs were fertilized with cryopreserved sperm. However, this result was achieved only in small-scale studies. As the hatchery techniques for greenlip and blacklip abalone have been mastered, a large scale evaluation of the performance of progeny produced with cryopreserved sperm should be conducted using parameters, such as fertilization rate, hatching rate, metamorphosis rate and the growth rate. Such evaluations have been conducted in Pacific oysters *Crassostrea gigas* (Adams et al., 2004) and blacklip pearl oysters *Pinctada margaritifera* (Hui et al., 2011). In finfish species, these types of assessments have been carried out in *Scophthalmus maximus* (Chereguini et al., 2001) and Psetta maxima (Suquet et al., 1998). Babiak et al. (2002) also found that the sperm collected from animals produced with cryopreserved sperm is less sensitive to cryopreservation treatment in rainbow trouts *Oncorhynchus mykiss*.

6. Furthermore, in order to effectively and safely operate sperm cryopreservation in commercial settings, detailed management protocols should be established. These protocols should be cost efficient, and include not only the standard procedures for sperm cryopreservation but also the supporting requirements such as personnel training, equipment calibration and verification, sperm storage maintenance procedures; sperm replacement strategies and data management (Tiersch, 2008; Kincaid, 2011; Li, 2012).

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APPENDIX A: SPERM CRYOPRESERVATION PROTOCOL FOR FARMED ABALONE IN AUSTRALIA

This protocol was developed by incorporating the results from my recent studies in farmed greenlip abalone (Liu et al., 2014a, b, in press b) and the protocol established by Li (2005) for wild greenlip abalone.

Materials and Equipments

1. dimethyl sulfoxide (DMSO), glucose, glycine (Figure 1) and 5μm filtered seawater.
2. autoclavable bottle, measuring cylinder, pipette, pipette tips, timer, slide, permeanent marker and conventional thermometer (0 to 100 °C) (Figure 1) and compound microscope, glass rod and cover slide.
3. 0.25 mL straw, goblet, cryocane and canister (Figure 1 and 4).
4. liquid nitrogen (LN) dewar for transporation with a pressurised liquid withdrawal device (Figure 2, a) and LN dewar for storage with canister (Figure 2, b).
5. styrofoam box with a specifically designed straw holding rack and protective full-face screen, latex glove and cryoglove (Figure 4).
6. abalone spawning system, spawning rack and sperm collection container (Figure 3).
7. water bath or water heating device and 10 L container for thawing, and 10 L container with 16 °C seawater for blacklip abalone or 18 °C seawater for greenlip abalone, which will be used for post-thaw sperm recovery (Figure 6, b).
8. refrigerator, ice, autoclave, Chux® cloths and padlock.

Methods

1. Preparation for cryopreservation

    1) Fill the transportation dewar with LN and install the liquid withdrawal device on the dewar at least one day in advance.

2) Check the saturation level of LN in the storage dewar by observing the calm surface of the LN (no bubbling) and fill the dewar if the canisters are not be covered by the LN.

3) Autoclave the necessary containers and utensils.

4) Prepare 12% DSMO + 2% glucose + 1.2% glycine stock solution for farmed greenlip abalone and 12% DSMO + 4% glucose for farmed blacklip abalone on the same day and keep in a refrigerator prior to use.

5) Write an ID number on the top of the desired cryocanes.

6) Put straws, goblets and cryocanes in a refrigerator.

7) Ensure the microscope, adjacent bench space and other utentials, such as spawning rack(s), sperm collection containers, torch, slides, cover slides, low temperature meter, pipette and its tips are ready to use.

8) Put on the cryogloves and full-face screen and fill the styrofoam box (with the straws holding rack inside) with the LN to the level of 2 ~ 3 cm using LN withdrawal device (Figure 5, a).

2. Sperm collection

9) Place one male broodstock in a spawning tank and adopt the combination of temperature increase and ultraviolet (UV) treatment to the spanwing induction (Figure 3, a).

10) When a male starts to spawn turn off the UV treated water and turn on the untreated water of the same temperature to the tank with the time recorded.

11) Turn off the fresh seawater supply and empty the tank 5 to 10 min later when spawning peaks (high frequency of sperm release).

12) Dry the abalone with a paper towel and turn the animal upside down by adjusting the position of the tank (Figure 3, c). If the animal falls off the tank put the animal on a rack with its shell facing down (Figure 3, b). Then put a shallow container underneath the animal.

13) Repeat steps 10 to 12 until enough sperm is collected from at least 3 individuals within a short period (40 min is recommended).

14) Check the motility of sperm collected from individual males under the microscope.

15) Pool the same volume of sperm together from different individuals with motility above 80% and stir gently by hand.

16) Add stock solution to sperm at a 1:1 ratio (v/v) to a final concentration of 6% DMSO + 1% glucose + 0.6% glycine (farmed greenlip abalone), 6% DMSO + 2% glucose (farmed blacklip abalone) and 1.6 x 10^8 sperm mL^{-1}, respectively and agitate the mixture quickly to make sure the solution is mixed with sperm properly.

17) Transfer 0.25 mL of sperm-chemical mixture into a straw and seal the straw.

18) Repeat steps 16 to 17 until the required numbers of straws have been filled.

3. Freezing procedures

19) Refill the styrofoam box with LN by the method described in steps 8.

20) Put the straws on the rack and start the timer.

21) After 10 min exposure to LN vapour, transfer straws into goblet that is attached to the cryocane wearing the rubber gloves.

22) When a goblet is full, or straw transfer is completed, put the cryocane into a canister by first removing the lid from the dewar, lifting the canister from the dewar to its mouth, putting the cryocane in and then return the canister back into its original position in the dewar (Figure 5, b).

23) Repeat steps 21 to 22 until all the straws on the rack have been put into the storage dewar.

24) Lock the storage dewar(s) with a proper padlock (Figure 6, a) and store the dewar in a safe and controlled place.

4. Long-term storage in liquid nitrogen

25) Check the LN level in the storage dewar once a week initially. Refill the dewar with LN when the liquid in the dewar drops below half.

26) Count the days between fillings and refill the dewar according to this interval from then on.

5. Preparation for thawing and fertilization

27) Mix the boiled and ambient seawater to produce 60 °C thawing water bath in a 10 L bucket with the help of a glass rod and a conventional thermometer.

28) Fill a 10 L bucket for recovery water bath with 16 °C filtered seawater for blacklip abalone or 18 °C for greenlip abalone.

29) Ensure the microscope and adjacent bench space are ready for use.

30) Get the other utensils, such as spawning facilities, eggs collection screens and containers, slides, cover slides, glass pipettes, pipette and its tips ready.

31) Put on the cryogloves and protective full-face screen.

6. Thawing and fertilization procedures (two people will be required if a large number of straws need to be processed simultaneously)

32) When the fresh eggs are collected from at least three females, thawing and recovery water baths and the filtered seawater at the required temperature are ready, take the cryocane with the desired ID from the storage dewar by lifting the holding canister to the dewar mouth, taking the cryocane from the canister, turning the canister back to its original position in the dewar.

33) After taking all the straws from the goblets, put the straws into a 60 °C water bath immediately.

34) Transfer the straws from the thawing water bath to a recovery water bath as soon as the ice in the straws melts.

35) At the same time a second person can repeat steps 33 and 34 if more straws are needed for fertilization.

36) Put the collected eggs into a container and wait for the eggs to settle on the bottom.

37) Check the the sperm motility from randomly selected straws under a

light microscope.

38) Using a pipette transfer the concentrated eggs from the bottom of the container to the fertilization container(s).

39) Fertilize the eggs by emptying the straws into the fertilization container directly and stirring the container regularly using hand for mixing.

40) Start the timer at the same time.

41) Wash the eggs on the screen after 10 min fertilization.

42) The fertilized eggs will then be maintained in the same way as those produced with fresh gametes.

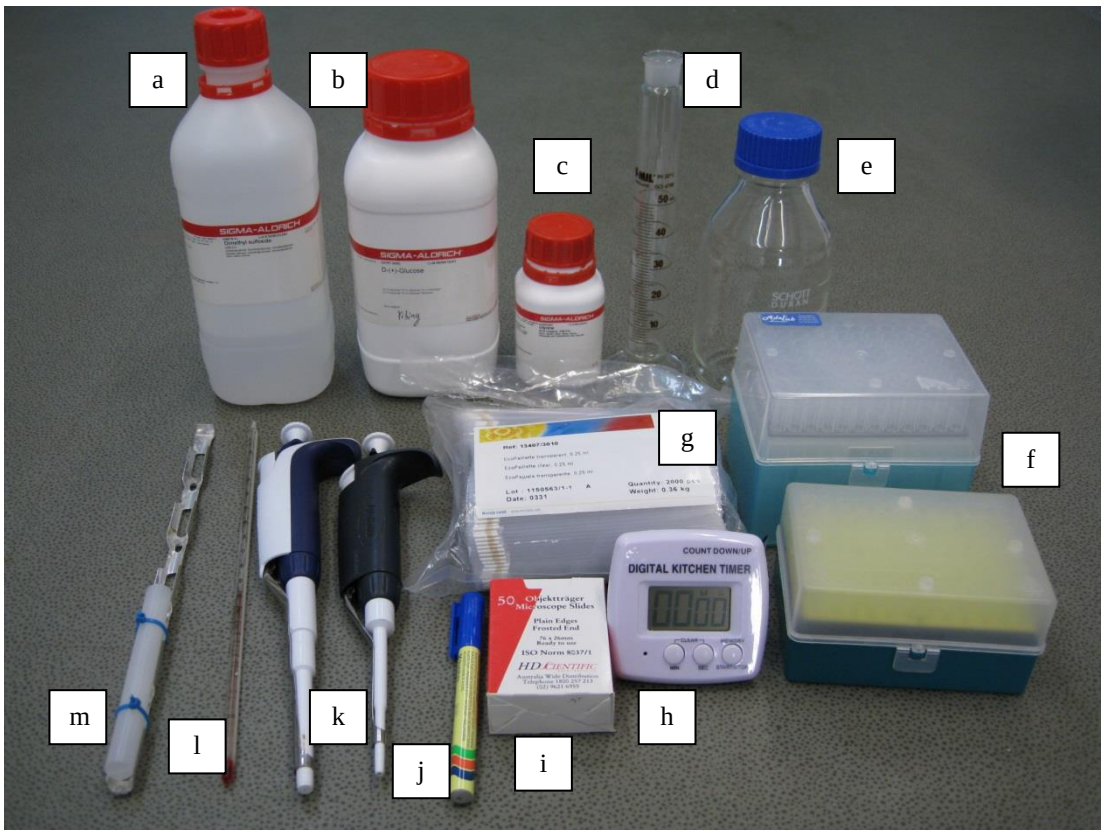

Figure 1. a) DMSO; b) glucose; c) glycine; d) measuring cylinder; e) autoclavable bottle; f) pipette tip; g) 0.25 mL straw; h) timer; i) slide; j) permanent marker; k) pipette; l) converntional thermometer and m) goblet attached to cryocane.

Figure 2. a) liquid nitrogen transportation dewar and b) liquid nitrogen storage dewar.

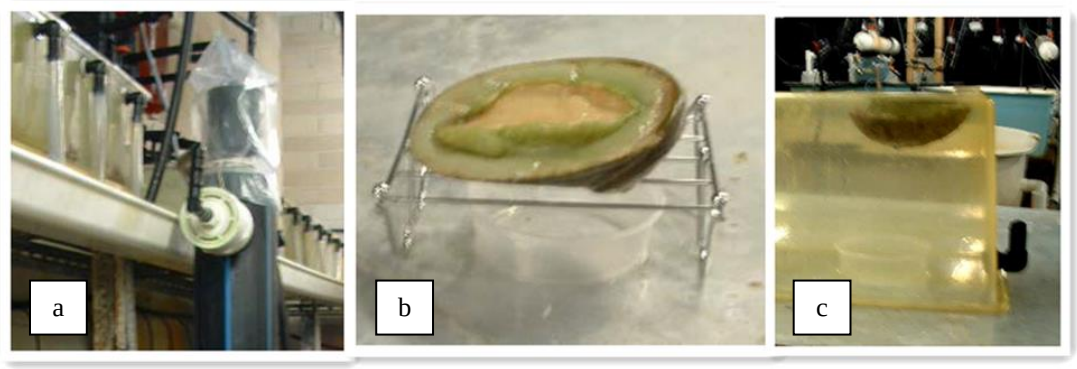

Figure 3. a) abalone spawning system; b) sperm collection with a rack and c) sperm collection.

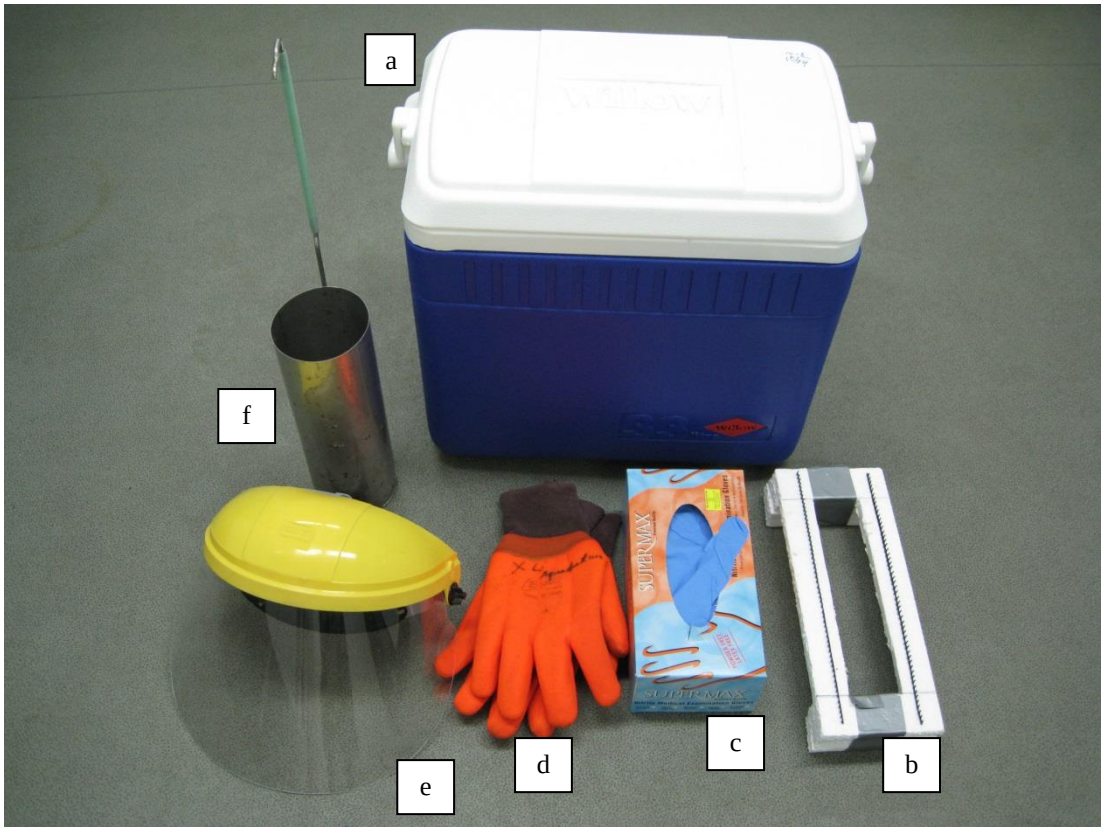

Figure 4. a) styrofoam box; b) straw holding rack; c) latex glove; d) cryoglove; e) protective full-face screen and f) canister.

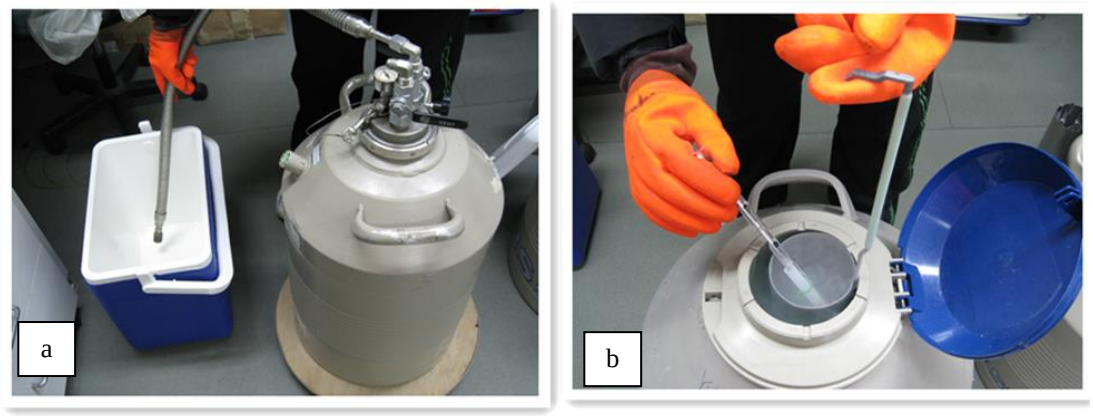

Figure 5. a) fill a styrofoam box with liquid nitrogen and b) transfer cryocane (with goblet containing straws) in canister and store the canister in storage dewar.

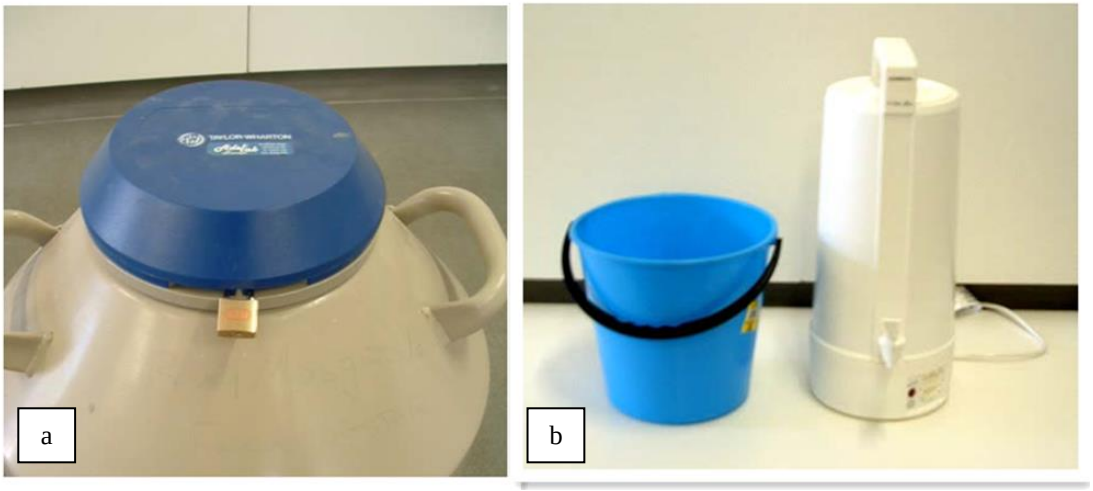

Figure 6. a) lock up the storage dewar and b) thawing equipment.

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