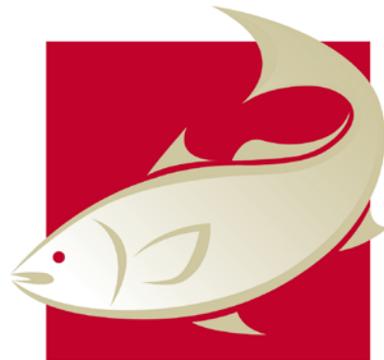


Scope of Options to Establish Gamete Cryobanking Services to Genetic Improvement Programs in Australian Aquaculture Industry

Xiaoxu Li

Project No. 2008/773



**AUSTRALIAN
SEAFOOD
COOPERATIVE
RESEARCH CENTRE**

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2. NON-TECHNICAL SUMMARY:**2008/773 Scope of options to establish gamete cryobanking services to genetic improvement programs in Australian aquaculture industry.**

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OBJECTIVES:

1. To review the short, medium and long term requirements of gamete cryobanking for each Australian Seafood Cooperative Research Centre (AS CRC) partner investing in genetic improvement.
2. To review the system requirements according to existing cryopreservation protocols, expected sample sizes, quality control measures and management strategies.
3. To identify the technical gaps in each priority species.
4. To identify the best or the most practical option(s) to develop gamete cryobanking services for genetic improvement programs in the AS CRC.

OUTCOMES ACHIEVED TO DATE

1. Identified that sperm cryopreservation techniques have been published for all species investigated in this study (Atlantic salmon, barramundi, tiger prawn, Pacific and Sydney rock oysters, greenlip and blacklip abalone, pearl oyster). Oocyte cryopreservation techniques have been published in Pacific oysters only.
2. Developed a model to estimate the cost of establishing an onsite cryobanking service (both cryopreserving and storage) with back up storage off site that can be used as a tool to compare and select cryobanking options that would best suit the industries short and long term needs.

3. Identified the key technical gaps in the cryobanking of aquatic species as absence of quality control measures in protocols with published results documenting mean results only, and lack of techniques critical to reconstruct the breeding nucleus.
4. Recommended a stepwise approach as the most practical method of establishing gamete cryobanking services for genetic improvement programs in Australia, noting that a new independent facility should not be ruled out if required funding is available.

The first successful sperm cryopreservation was published in 1949 by Polge and his colleagues in livestock. Similar progress was made a few years later in an aquatic species (Blaxter 1953). Since then livestock sperm cryopreservation has developed into a billion-dollar industry globally (Tiersch 2008), evolving from breeder's clubs and public cooperatives providing artificial inseminations (Herman 1980, Caffey & Tiersch 2000), while sperm cryopreservation in aquatic species remains a research activity with little commercial application (Tiersch 2008). Although a similar industry has been anticipated for aquatic species (Caffey & Tiersch 2000) the overall cryopreservation process needs to be improved, and the approach needs to be integrated into an efficient large-scale platform for it to become a reliable, cost-effective business (Tiersch 2008).

This project focused on scoping the options available to establish a cryobanking service for genetic improvement programs in the AS CRC. This involves cryopreserving a small amount of gametes from the top performing individuals as required by individual breeding programs to form the breeding nucleus. This approach therefore differs from that for purely commercial applications.

The first step in assessing cryopreservation options for the aquaculture industry was to establish the short, medium and long term requirements of gamete cryobanking for each AS CRC partner investing in genetic improvement programs. Discussions with relevant industries revealed that:

-
- Ideally, the proposed cryobanking service should assist both commercial hatcheries and selective breeding programs in all industry sectors surveyed, noting that this project focused on the selective breeding programs only.
 - All selective breeding programs expected their replacement strategy to be generation by generation.
 - Maintaining genetic diversities through cryobanking has not been considered a priority at this stage of breeding program development.
 - The short and long term requirements for numbers of males and female broodstock are the same, ranging from 100 individuals per sex in Pacific oysters to 300 in Sydney rock oysters.
 - All the breeding programs expect ownership of their germplasms stored off site.
 - The breeding programs have limited, if any, equipment required for cryopreservation and cryostorage.
 - All programs, except for Pacific oyster, indicated they have trained staff who can conduct cryopreservation on-site.
 - No specific cryopreservation data management system exists across any of the breeding programs.
 - Sperm quality is evaluated in the industry by visual motility assessment and fertility examination.
 - Sperm cryopreservation protocols exist in all species surveyed, while oocyte cryopreservation protocols were published for Pacific oysters only.

The system requirements were then assessed according to existing cryopreservation protocols, expected sample sizes, quality control measures and management strategies. A review of published protocols for sperm or spermatophore cryopreservation in salmon, barramundi, prawns, abalone, pearl oysters, Sydney rock oysters and Pacific oysters and oocyte cryopreservation in Pacific oysters demonstrated that these protocols are highly variable even within the same species. The only reliable quality control measure that can be applied across protocols is assessing fertility. This requires both male and female gametes present at the same time for assessment and needs to be taken into account when establishing a cryobanking service.

A model, based on the published protocols for the cryopreservation of aquatic gametes was developed to estimate the costs associated with an on-farm cryobanking service (both cryopreserving and storage, with an off-site back-up) that can be compared against other available options. The model estimates the costs associated with cryobanking a family unit of sperm (sufficient sperm to establish a full-sib family) in the selective breeding program, and the relative costs associated with changes in the nucleus population size (number of families) for each of the four species groups investigated; finfish (salmon and barramundi), prawn (tiger), bivalve (pearl oyster, Pacific oyster and Sydney rock oyster) and abalone (greenlip and blacklip). The model provides selective breeding programs with a basic tool to compare and select the cryobanking options that would best suit their short and long term needs. Model results indicate that a cryobanking service established at a commercial hatchery with a back up cryostorage at a research or existing cryobanking facility would:

- Have an initial capital investment of approximately \$71,416.
- Cost between \$2.43 and \$0.85 in freezing materials to cryopreserve the “family unit” or per male based on the biological parameters and the storage system configurations defined in this study.
- Store sperm from a maximum of 360 males per 35L dewar, as defined in this study.
- Have relatively consistent cryopreservation costs per family unit (per male) with changes in the breeding nucleus size from 50 to 400 full-sib families for each species group considered. However, costs increase 3 fold in species requiring natural spawning such as abalone, compared with those where sperm is collected using strip-spawning such as salmon, prawns and oysters.
- Have decreasing annual maintenance costs per family unit (per male) with increases in the breeding nucleus size from 50 to 300 full-sib families per generation, ranging from approximately \$260 to \$43 per male per year respectively.
- Theoretically produce up to 607,500, 84,375,000 and 3,375,000 fertilised eggs in the 0.5mL straws stored in a 35L dewar for finfish, bivalve and abalone groups, respectively, according to the biological parameters and cryostorage equipment configurations used in this study.

One of the key components of this project was to identify the current technical gaps that would need to be overcome to successfully establish a cryobanking service for aquatic breeding programs. A review of published protocols identified the need for quality control measures to ensure desired outcomes are achieved as the published results do not report variation in mean results and cannot be predicted or accounted for in models. Secondly, research and development (R&D) is needed to develop techniques critical to reconstructing the breeding nucleus. Pacific oysters is the only species in which both male and female gametes have been successfully cryopreserved, although there are no published results on the successful reconstruction of the breeding nucleus solely from cryopreserved gametes for any aquatic species. This must be overcome to fully secure the genetic variations established in breeding programs.

After reviewing the current status of cryopreservation in aquatic species and the facilities and expertise available in Australia, the project recommends that the most practical option for developing gamete cryobanking services for genetic improvement programs in the AS CRC would be a stepwise approach involving a research or an existing cryobanking facility. This approach would provide the service required but also addresses the issues and gaps raised. Key steps should include:

- Establishing a project to secure the required equipment.
- Optimisation and standardisation of published protocols using the equipment available to the breeding program and the current on site hatchery production system.
- Establishing a training program for procedural efficiency and personnel recruitment.
- Funding research and development to fill technical gaps identified.
- Periodically reviewing the long-term needs of the selective breeding program in the Australian aquaculture industry.

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4. BACKGROUND

Physical acquisition of genetic material is relatively quick and provides an important reserve of genetic resources that can be used for a wide variety of conservation and research interests (McClintock *et al* 2007). Advances in cryogenic storage techniques in livestock led to the establishment of cryobank facilities that can store live biological materials in liquid nitrogen (-196°C) for hundreds of years (Lueng 1991).

Cryopreservation techniques are specific for both species and material types (sperm, oocyte, embryo, tissue and organ). For example, cryobanking has been used commercially for many decades in livestock genetic improvement programs. So far, however, the cryopreservation technology developed has not been sufficient to yield reliable fertility following artificial insemination in commercial swine production systems (Bailey *et al* 2008). Cryopreservation of semen is possible in all domestic animals and deep-freezing of mature oocytes for prolonged periods prior to in vitro fertilization is possible in some mammalian livestock species. Cryopreservation techniques have also been developed in medicine with preservation of human semen and oocytes (and embryos) to assist reproduction.

Cryobanking in association with reproductive biotechnologies such as artificial insemination and embryo transfer have assisted livestock improvement programs increase the rate of genetic progress and disseminate the contribution of genetically superior parents to the population and reduce the risk of disease transmission (Bailey *et al* 2008).

Currently only limited generic commercial cryobanking services are available for aquatic animal species (Lang *et al* 2003, Greer & Harvey 2004, Dong *et al* 2007, Cryogenetics). Sperm cryopreservation in aquatic species have been improved substantially in the last couple of decades, with sperm in many finfish and shellfish species having been cryopreserved with varying levels of success (Gwo 2000, Tiersch 2000). The development of cryopreservation methods for oocytes and embryos in aquatic species is challenging. After many decades of research the successful cryopreservation of eggs, embryos and larvae has only been documented in four bivalve species and one finfish species (Renard 1991, Xue 1994, Usuki *et al* 1999,

Paniagua-Chavez and Tiersch 2001, Tervit *et al* 2005, Chen & Tian 2005, Liu & Li 2008, Wang *et al* 2011).

Development of cryopreservation techniques and the establishment of a cryobanking service centre for the Australian aquaculture industry will facilitate genetic improvement of economically important traits in the breeding program; disseminate these improvements across the population; maintain genetic diversity; and protect breeding programs from epidemic diseases.

The major barriers inhibiting the establishment of cryobanking services for aquaculture are: 1) high start up and maintenance costs; 2) the relatively small size of individual aquaculture sectors; and 3) the lack of standardised cryopreservation protocols. The development of a specialised cryobanking centre, proven to be effective and practical that could be used by all aquaculture breeding programs in Australia, is likely to overcome the first two barriers. While there would be advantages in using existing specialised cryobanking centres that has access to standardized procedures and expensive equipment (Dong *et al* 2007) further evaluation would be required to assess the suitability for aquatic species.

Some promising results have been reported using commercial dairy cryobanking facilities to cryopreserve sperm from a couple of aquaculture species (Lang *et al* 2003, Dong *et al* 2007). However, it is not clear whether the freezer settings at the cryobanking centres and gamete transportation methods used in these studies would suit other aquatic species. An alternative option would be to conduct on-farm cryopreservation and then send the frozen gametes to the specialised centre(s) for storage. This option would require highly skilled on-farm technicians and might not be practical for processing large numbers of samples. In addition, the standardised quality control measures may be difficult to implement because specialised equipment may not be available at commercial hatcheries.

The first crucial step towards establishing a gamete cryobanking service for priority aquatic species in Australia was to evaluate all potential options through a desktop study and on-site visits.

5. NEED

Given the small population of aquaculture stocks, maintenance of genetic diversity is a critical issue for many aquaculture industries. Genetic diversity within a population increases its ability to sustain the population in case of disease outbreaks and environmental changes. A sufficient level of genetic diversity is also essential for long-term improvement of economically important traits.

There is also a strong need to protect existing breeding programs from epidemic diseases. For example, the outbreak of abalone viral ganglioneuritis in Victoria in 2006, forced farms involved in the selective breeding program to destock, resulting in the loss of the breeding nucleus and more than seven years effort. Had a cryopreservation program been in place to freeze gametes from selected individuals, much of the progress made would have been recoverable.

Gamete cryopreservation is a secure method for the ex-situ preservation of genetic diversity and genetically improved materials, thus providing opportunities to reconstruct the original genetic make-up, re-establish the improved nucleus population, and establish genetic linkage among different generations and/or runs. Moreover, transporting cryopreserved gametes is relatively simple, has less chance for disease translocations, as gametes are less likely to carry pathogens than whole animals, and enhances the efficiency of disseminating genetic gains to industry.

Using existing specialised cryobanking facilities would be one way of providing the aquaculture industry with immediate access to cryobanking services. However, as gamete cryopreservation protocols for aquatic animals are species specific, the suitability of standardised procedures used at these centres needs to be evaluated for aquatic species. The use of existing protocols and related issues needs to be assessed before a practical method of establishing gamete cryobanking services for priority aquatic species in Australia can be recommended.

6. OBJECTIVES

1. To review the short, medium and long term requirements of gamete cryobanking for each Australian Seafood Cooperative Research Centre (AS CRC) partner investing in genetic improvement.
2. To review the system requirements according to existing cryopreservation protocols, expected sample sizes, quality control measures and management strategies.
3. To identify the technical gaps in each priority species.
4. To identify the best or the most practical option(s) to develop gamete cryobanking services for genetic improvement programs in the AS CRC.

7. COST ANALYSIS OF SPERM CRYOPRESERVATION IN DIFFERENT SPECIES GROUPS

The production unit costs for integrating cryopreservation into an existing fish hatchery was analysed by Caffey & Tiersch in 2000 based on generic activities required for fish sperm cryopreservation and the maximum storage capacity of a dewar being 3000 straws. In that analysis the production unit was defined as a single 0.5 mL plastic straw and costed according to the business model applied in USA, with specific focus on the differences between public and private investment scenarios, as there is no sale tax on public institutions. To assist in the identification of the best or the most practical option(s) to develop gamete cryobanking services for genetic improvement programs in the AS CRC, the costs for provision of a cryobanking service need to be further divided into the components required for cryopreservation and cryostorage and their sensitivities to changes in the size of breeding nucleus, especially the number of broodstock used. It is anticipated that this number will vary among species and within species with the development of the breeding program, especially for new programs.

7.1. Cryopreservation techniques in aquaculture species

Sperm cryopreservation for most aquaculture species has been developed based on two types of techniques. The first technique, referred to as the “uncontrolled rate freezing method” in this report, controls the predetermined end temperature and is performed either in liquid nitrogen vapor (Tsai & Chao 1994, Li 2004, Jodun *et al* 2006, Lahnsteiner & Mansour 2009), a methanol bath chilled to ~ -70°C with dry ice (Adams *et al* 2009) or on dry ice (the so called pellet freezing method) (Dziewulska *et al* 2011). When liquid nitrogen vapor is used to freeze, a self constructed insulated box such as those converted from an esky (Tsai & Chao 1994, Li 2004, Lahnsteiner & Mansour 2009) or a nitrogen vapor shipping dewar (Jodun *et al* 2006) is used. The second technique, referred to as the “controlled rate freezing method” in this report, controls both freezing speed and the end temperature, and needs a purpose-built freezing machine. The second technique has been used successfully to develop an oocyte cryopreservation protocol in Pacific oysters (Tervit *et al* 2005). In aquatic

species, the gamete cryopreservation procedures can be described by the following six key steps (Figure 1).

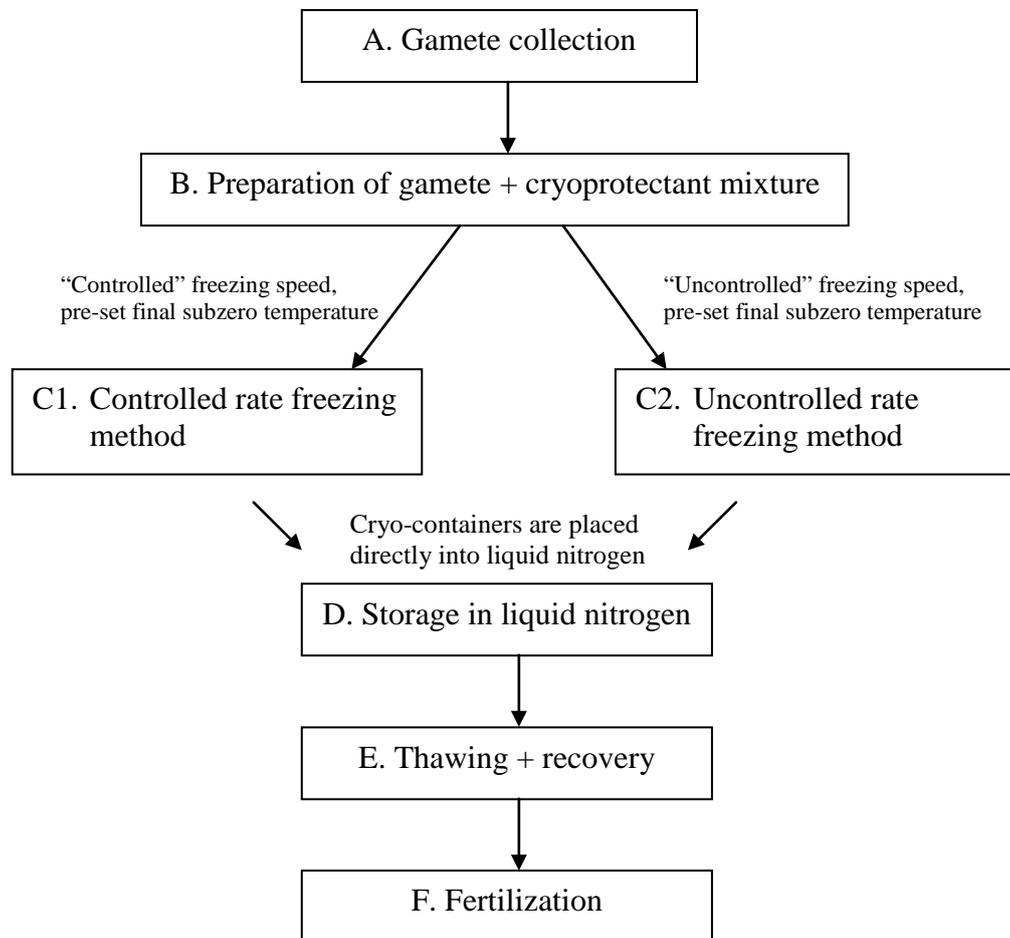


Figure 1. Key steps in gamete/embryo/larval cryopreservation in aquatic species

Protocols for sperm cryopreservation have been developed and published for: Atlantic salmon and closely related species (Stoss & Refstie 1983, Alderson & Macneil 1984, Cloud *et al* 1990, Yamano *et al* 1990, Gallant *et al* 1993, Lahnsteiner *et al* 1997, Dreanno *et al* 2000, Babiak *et al* 2002, Jodun *et al* 2006, Negus 2008, Sandilands *et al* 2009, Lahnsteiner & Mansour 2009, Dziewulska *et al* 2011); greenlip and blacklip abalone (Li 2004, 2005, 2009); Pacific oysters (Adams *et al* 2009, Dong 2009); Sydney rock oysters (O’Conner, pers. comm.); and prawns including tiger prawns (Anchordoguy *et al* 1988, Akarasanon *et al* 2004, Vuthiphandchai *et al* 2005, 2007, Bart *et al* 2006, Alfaro *et al* 2007, Liao *et al* 2008, Damrongphol & Akarasanon 2009, Salazar *et al* 2009). Investigations on the development of cryopreservation techniques

have been published in pearl oysters (Lyons *et al* 2005, Acosta-Salmon *et al* 2007, Kawamoto *et al* 2007, Narita *et al* 2008, Hui *et al* 2011) and barramundi (Leung 1987, Palmer *et al* 1993). A protocol for oocyte cryopreservation has been established in Pacific oysters (Tervit *et al* 2005).

7.2. Species comparison of components within key cryopreservation steps for selective breeding programs in Australia

Cryopreservation protocols differ substantially both within and between species. For example, a protocol developed by Dong *et al* (2009) used either a 0.25 or 0.5 mL straw to freeze Pacific oyster sperm using the controlled-rate freezing method, whereas Adams *et al* (2009) developed a onestep protocol to freeze 4.5 mL vials in a methanol bath cooled to -70°C with dry ice. The question is which protocol is more suitable for the provision of a cryobanking service for selective breeding programs. To determine this many factors need to be considered including specifications of the proposed facilities for the maintenance of families, as well as mating design (full-sib vs half sib), sperm to egg ratio, etc.. Some of this information is confidential and will be assumed in this study.

A species comparison of critical cryopreservation steps and related references is provided in Table 1.

Table 1. A species comparison of components within key cryopreservation steps for aquatic breeding programs

Step	Salmon	Barramundi	Prawn	Abalone	Pearl oyster	Sydney rock oysters	Pacific oysters	
	sperm	sperm	spermatophore	sperm	sperm	sperm	sperm	eggs
A. Gamete collection								
extender	specifically formulated	Ringer's solution	Ca-free seawater	filled seawater	n/a	n/a	n/a	n/a
cryoprotectant agent (CPA)	multiple CPAs	single CPA	single CPA	single CPA	2 CPAs in distilled water	2 CPAs in distilled/Milli-Q water	single CPA in filled seawater or Ca-free Hanks' or 2 CPAs in distilled water	single CPA in Milli-Q water
activation solution	specifically formulated	seawater	n/a ¹	not needed	seawater	seawater	Seawater or Hanks'	n/a
hermaphrodite		sequential from male to female		simultaneous at very low frequency	simultaneous at very low frequency	simultaneous at very low frequency?	simultaneous at very low frequency; can change gender status between spawning seasons	
spawning method	stripped by abdominal massage	stripped by abdominal massage	removed with forceps	air explosion; temperature shocks; UV treated water	stripped with a destructive method	stripped with a destructive method	stripped with a destructive method	
sperm centrifugation	n/a	n/a	n/a	no, if	n/a	n/a	n/a	n/a

				spawned "dry"; yes if in water				
temperature	2 - 4°C	on ice	room temperature	on ice	~4°C; room temperature (22°C)	on ice	on ice	5°C
spawning ratio	n/a	n/a	variable	highly variable	n/a	n/a	n/a	n/a
quality level recommended or used	≥70% motility	>90% motility	not melanised; >90% viability (cell morphology or staining)	~100% motility	> 95% motility		> 95% motility	n/a
holding period prior to cryopreservation	4 h	3-4 h		< 2 h			a few days	up to 4 days
B. Preparation of gamete and cryoprotectant mixture								
equilibration duration	< 2 min 45 s	10-15 min	30 min	< 3 min	< 10 min	~10 min	5 min	20 min from addition of CPA solution
final gamete concentration	~1X10 ⁹ /mL	1:4 dilution	n/a	~5X10 ⁸ /mL	~6X10 ⁷ /mL		~1X10 ⁹ /mL	10 ⁶ /mL
freezing containers	0.25 or 0.5 mL straws; 0.1 – 0.2 mL pellets;	1-2 mL vials	1.8 mL vial	2 mL vial	0.25 or 0.5 mL straw	0.5 mL straw	0.25 or 0.5 mL straw; up to 4.5 mL vials	0.25 mL straw
C. Freezing								

methods	programmable machine; liquid nitrogen vapour; on dry ice blocks	methanol/dry ice bath	programmable machine	liquid nitrogen vapour	liquid nitrogen vapour	liquid nitrogen vapour	liquid nitrogen vapour; methanol/dry ice bath; programmable machine	programmable machine
D. Storage in liquid nitrogen								
quality checking of frozen gamete/spermatophore	> 5% motility		n/a	>30% motility	86% motility (the highest)		> 50% motility	
E. Thawing and recovery								
temperature	low (10-11°C); high (40°C)	30°C	30°C	50°C	27°C	25°C	40°C or room temperature	28°C
thawing duration	30 s (low temperature); 7 s (high temperature)	2-3 min	3 min	as soon as ice melted	15 s	as soon as ice melted	6-7 s for straw at 40°C; as soon as ice melted for vials at room temperature	as soon as ice melted
recovery period	immediate fertilisation	immediate fertilisation		< 5 min	immediate fertilisation	immediate fertilisation	< 10 min	60 min
F. Fertilization								
fertilisation capacity	~225 eggs per 0.5 mL straw	$\geq 1.2 \times 10^5$:1 sperm:egg	n/a	2×10^4 :1 sperm:egg	1×10^4 :1 sperm:egg	$1-5 \times 10^3$:1 sperm:egg	$> 2 \times 10^3$:1 sperm:egg	~20:1 sperm:egg

	ratio		ratio		ratio		ratio		ratio
specific treatments	egg hardening with iodophore solution after fertilisation		implanted into the thelycum of newly molted female	washing fertilised eggs before jelly layer melted	NH ₃ induced oocyte maturation				very slow cooling rate (-0.3°C/min) from -10 to -35°C; addition and removal of CPA solution in steps

Note:

	quick action is required at most steps	evaluation until hatched larvae	viability declined after >60 days in liquid nitrogen; multiple spermatophore collection; evaluation until hatching stage	the protocol was developed with mature broodstock directly from wild; evaluation on resultant larvae	the protocol was developed with stripped gametes; 1 h fertilisation period; initial sperm motility highly variable between individuals; evaluation on resultant larvae	the protocol was developed with stripped gametes; evaluation on resultant larvae	the protocol was developed with stripped gametes	the protocol was developed with stripped gametes; highly variable in freezability among females
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References

Dziewulska <i>et al</i> 2011; Lahnsteiner & Mansour 2009;	Palmer <i>et al</i> 1993; Leung 1987.	Damrongphol & Akarasanon 2009; Salazar <i>et al</i> 2009;	Gwo 2000; Gwo <i>et al</i> 2002; Li 2005; Salinas-	Hui <i>et al</i> 2011; Acosta-Salmon <i>et al</i> 2007;	O'Conner pers. comm.	Li 2009; Dong <i>et al</i> 2009; Adams <i>et al</i> 2009	Tervit <i>et al</i> 2005
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Negus 2008; Jodun <i>et al</i> 2006	Vuthiphandchai <i>et al</i> 2007; Bart <i>et al</i> 2006	Flores <i>et al</i> 2005	Lyons <i>et al</i> 2005
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¹. Not applicable.

Key factors within each step that will affect the development of a cryobanking service for aquaculture species in Australia are summarised as follows:

A. Gamete collection

1) Purpose of the proposed cryobanking service:

Cryobanking can serve different purposes within genetic improvement programs, including: distribute genetic gains to the industry; assist in establishing the breeding nucleus and genetic linkages; and/or preserve genetically improved materials to minimise the impacts of potential catastrophes and disease outbreaks.

2) Candidates for gamete cryopreservation:

Suitable candidates are normally determined by their breeding values, calculated according to the method and parameters established in the respective breeding programs.

3) Methods used for gamete collection:

Two main methods are used in the cryopreservation protocols: strip or natural spawning. The strip spawning method can be further divided into “non-destructive strip spawning” method (salmon, barramundi and tiger prawn) and “destructive strip spawning” method (Pacific oysters, Sydney rock oysters and pearl oysters). Abalone are normally induced to spawn naturally by manipulation of water temperature, addition of UV treated water, etc.

4) Sex determination and sex change:

All species included in this scoping investigation can be sexed by non-destructive methods. Only Barramundi and Pacific oysters are known to change genders over their lifespan with Barramundi maturing as males first and then changing to females later on (Moore 1979) and Pacific oysters are able to change sex in both directions although sex ratios are skewed from male to female in the younger and older groups respectively.

5) Anticipated spawning ratio:

Based on published protocols spawning ratios are only relevant to abalone as they are the only species to spawn naturally. The ability to cryopreserve sperm allows designed mating to be achieved as the exact timing of spawning cannot be predicted. This will also become relevant to those

species currently using the “destructive strip spawning” method for gamete collections. Ideally non-destructive techniques need to be developed for them so that genetic gains that have been achieved in the top performing individuals can be retained for commercialisation.

6) Timing for gamete collections:

It is expected that the individuals used for gamete cryopreservation would be those selected to establish the breeding nucleus for the next generation, serving the dual purpose of providing gametes for both cryopreservation and production of the next generation. In this instance cryopreservation would be conducted at the same time as new families were being established.

7) Gamete quality assessment methods:

Methods for determining sperm quality varied among protocols, including assessing the motility and percentage of vigorous sperm (assessed under a compound microscope); calculating the ratio of living/dead sperm (assessed under a compound or fluorescent microscope following staining); computer-assisted sperm motility assessment; and fertility assessment etc.

8) Translocation issues:

These issues need to be considered for individual species to determine the appropriate location and associated costs for gamete collection and storage.

B. Preparation of gamete and cryoprotectant mixture

1) Number of cryo-containers (straws or vials) and replicates per candidate:

This would be determined for each candidate by the amount needed to produce the required number of progeny for a particular facilities allocation for that family and year class; the mating design (full-sib or half sib) used by the breeding program; and the number of performance evaluation facilities involved.

2) Individual and temporal variation in gamete qualities.

3) Individual variation with regard to their tolerance to cryopreservation processes:

Gametes from different individuals might respond to cryopreservation processes differently, resulting in lower fertility in some individuals.

C. Freezing

1) Equipment capacity:

There are a few companies producing commercially available controlled rate freezing machines. SARDI uses CryoLogic cryochambers with a maximum freezing capacity per run of twenty-three 0.5 mL, forty-six 0.25 mL straws, fifteen 1 mL, ten 2 mL or five 5 mL vials. The self-constructed freezing containers used for uncontrolled rate freezing methods have not been standardised although specifications have been provided in some protocols (Li 2005, Lahnsteiner & Mansour 2009).

2) Accessibility of liquid nitrogen:

Most aquaculture hatcheries in Australia are located in remote regions which may increase the cost of liquid nitrogen.

D. Storage in liquid nitrogen

1) Size of the breeding nucleus:

The nucleus size refers to the number of families that will be established each generation/year class, which will in turn determine the storage capacity required per generation and the related maintenance costs.

2) Generation cycle and replacement strategy:

It is anticipated that the replacement strategy would change with the development of the selective breeding program. The simplest strategy would be to replace all the materials with the storage of each new generation. Using this strategy, the replacement frequency is determined by the species reproduction cycle, for example two years in Pacific oysters. In this instance there would be a short overlapping period where more than one year class is stored at a time. However, some unique genetic materials may be preserved for more than one generation.

3) Quality assessment of frozen gametes:

One container from each run is assessed 4 hours after the samples have been frozen in liquid nitrogen to assess their quality and determine whether samples need to be replaced.

E. Thawing and recovery

1) Time when the cryopreserved gametes need to be at the hatchery:

Gametes must be used immediately after thawing. As such thawing must occur on site and coincide with the opposite sexes spawning. Resulting progenies will need to be reared in a suitable facility.

F. Fertilization

1) Post-thaw gamete fertilisation procedures:

Protocols for fertilising cryopreserved gametes normally differ from standard procedures used to fertilise fresh gametes. For example, Adams *et al* (2009) and Dong *et al* (2009) protocols recommend a sperm:egg ratio of 2000-10000:1 for cryopreserved Pacific oyster sperm, about 100 to 1000 times higher than the ratio normally used for commercial spat production using fresh sperm.

2) Mating design:

The mating design between male and female individuals will be determined by the selective breeding program in order to achieve the desired genetic gains.

3) Spawning:

Except for Pacific oysters, oocyte cryopreservation techniques are not available for aquatic species and therefore fresh oocytes need to be sourced. Currently, it is very challenging to predict when natural spawning will occur in species that are induced to spawn naturally, such as abalone.

In addition, the data recording system, especially the sample identification number used for gamete storage in liquid nitrogen should match the identification numbers created in the selective breeding program. The data management system for aquaculture selective breeding programs will be developed or determined by a separate AS CRC project.

To achieve the objectives of this AS CRC project, it is necessary to determine: 1) the cryopreservation costs associated with cryopreserving enough sperm to establish one family in the respective breeding program; and 2) related annual maintenance costs. This is critical in being able to compare the costs of the potential options available for establishing a cryobanking service for the aquaculture selective breeding programs in

Australia. The number of families considered in this project costs analysis is based on the predicted family numbers in the industry survey.

7.3. Cost analysis for establishing a cryobanking service at a hatchery involved in the selective breeding program

In this analysis and the subsequent comparison between different options, the family unit cost is used and defined as the cost required for the storage of enough sperm to establish a full-sib family in the next generation. It has two components: a) the cryopreservation costs; and b) the storage costs. The storage costs include costs incurred from the time gametes are put into the liquid nitrogen dewars to when they are taken out for family establishment in the breeding program. The cryopreservation (freezing) costs, on the other hand, include those required for activities during cryopreservation. Thawing and fertilisation costs are not included in this analysis as they are likely to be conducted on farms and would therefore have limited, if any impact on the outcomes of a comparison between options to establish a cryobanking service.

7.3.1. Materials and Methods

In this analysis the species listed in Table 1 are further grouped into finfish (salmon and barramundi), prawn (tiger prawn), bivalve (Pacific oyster, Sydney rock oyster and pearl oyster) and abalone (greenlip and blacklip) as the sperm cryopreservation techniques developed for the species within each group are similar. In addition, the sperm in all these species groups can be cryopreserved with the uncontrolled rate freezing method, using self constructed freezing equipment. The equipment used varies widely across studies; however, these costs are relatively small and most materials are easily accessible.

To simplify the assessment for establishment of a cryobanking service to the Australian aquaculture industry, the cost estimations are based upon the liquid nitrogen vapour freezing method with 0.5 mL straws (finfish, bivalve and abalone groups) or 2.0 mL cryovials (prawn group) as this method has been used in most

sperm cryopreservation studies and established protocols in aquatic species. In the capital investment estimations, the essential investments include minimal equipment to conduct the protocols developed with the liquid nitrogen vapour method. Optional equipment would be required to further improve the quality of cryopreserved gametes and refine existing protocols or to develop new protocols (Caffey & Tiersch 2000).

The key assumptions included in the cost estimations are as follows:

General

- 1) Costs for spaces required for gamete collections, cryopreservation and dewar storages are not included in this analysis as it is anticipated that they can be allocated from the existing infrastructure available on the farm.
- 2) The breeding program is managed on one farm and the distribution of genetic gains is achieved through the sale of progenies produced on the same farm.
- 3) The information provided in Tables 2-4 are all based on the breeding nucleus size of 100 full-sib families per generation, although a range from 50 to 400 families per generation have been analysed and presented in the results.
- 4) The purpose of cryopreserving gametes is for establishing the nucleus families only, not for linkage families (across generations and batches), genetic gain distribution, etc. It is anticipated that the inclusion of linkage families would have marginal effects on the costs estimated.
- 5) The number of individuals at different developmental stages is calculated according to survival rates between stages only. The suitability of existing facilities for maintaining a small number of progenies per family has not been considered. In some breeding programs such as salmon the progenies from different families of the same generation are pooled after fertilisation.
- 6) For each male, an additional set of straws/cryovials will be processed in the same way as the primary set, but stored in a separate dewar for back-up purposes.
- 7) The price of chemicals and equipment are as listed on either SIGMA or Genetics Australia's website (April 2011), unless otherwise specified.
- 8) The costs associated with maintaining a database specifically for a cryobanking service for selective breeding programs has not been included.
- 9) All costs are presented in Australian dollars and GST exclusive.

Initial capital investments (Table 2)

- 1) For security reasons, the backup gametes in each generation are stored in a separate dewar(s).
- 2) Equipment depreciations are calculated using a straight line method for 10 years with no salvage value.
- 3) A cost of \$4000 is added as miscellaneous expenses to cover specific items required in different species groups although it is recognised that costs for this component might vary substantially among species. For example, limited specific equipment would be required for groups that can be strip-spawned in comparison with abalone where a specialised UV system is used to induce spawning, which costs approximately \$4000. It should be noted that this system can also be used in commercial hatchery productions.
- 4) The initial investment is financed using a 5 year loan with an annual interest of 8%.

Costs analysis on freezing materials (Table 3)

- 1) The information used in this analysis is based on the model species in each species group; salmon in finfish group, tiger prawn in prawn group, Pacific oyster in bivalve group and greenlip abalone in abalone group.
- 2) The ratios between different freezing materials are: one 35L dewar holds six canisters; each canister holds thirty canes; each cane holds two goblets or five 2 mL cryovials and each goblet holds five 0.5 mL straws. However, the costs for both dewar and canisters are not accounted for here as they are included in the initial capital analysis.
- 3) To minimise cross-contamination between sperm from different individuals and for easy operating, straws with sperm from the same individual will be held in the same goblet. Therefore, some goblets would not be full as less than 5 straws will be needed to establish a full-sib family in that species group.
- 4) The minimum egg numbers required at fertilisation are calculated according to the assumed survival rates at different development stages, the number of eggs used for one straw of sperm and the number of individuals required for performance evaluation during grow out per family.

Cost analysis of cryopreservation and annual maintenance (Table 4)

- 1) The breeding nucleus is replaced generation by generation, that is annually for prawns, every two years for bivalves, and every three years for abalone and finfish. It is also assumed that the new gametes would be processed before the previous generation is discarded.
- 2) The number of male broodstock per freezing run is determined by: a) a maximum of 30 straws/cryovials frozen per run (standardised across the self constructed freezing equipment); and b) a maximum of 5 male broodstock per freezing run to minimise the sperm aging effect.
- 3) Three litres of liquid nitrogen will be needed per freezing run.
- 4) A set of cryopreservation chemicals must be used within three years and therefore can preserve between two to four generations depending on the species.
- 5) Two technicians at 10% Full Time Equivalent (FTE) are required to cryopreserve sperm from 300 strip spawned broodstock (finfish, prawn and bivalve groups) or 100 natural spawned broodstock (abalone group).
- 6) The calculated capital depreciation for the cryopreservation period assumes that sperm from 300 strip spawned males or 100 naturally spawned males can be cryopreserved per month.
- 7) It is anticipated that thawing will occur on site, generally at a hatchery, to be used immediately. It is therefore independent of the cryobanking service provided and is not included in the analysis.
- 8) It is assumed liquid nitrogen will be refilled monthly, although it should be noted that it will evaporate at different rates depending on the air temperature surrounding storage dewars.
- 9) One technician at 5% FTE is allocated to maintain two storage dewars.
- 10) A contingency of 2.5% of cryopreservation costs or annual maintenance costs is included in the respective sections.

7.3.2. Results and discussion

This report details for the first time the costs per single male broodstock (full-sib family) associated with sperm cryobanking services for Australian aquaculture species in selective breeding programs. Reported costs are further divided into cryopreservation and annual maintenance costs. These estimates provide an economic basis for assessing the best strategies to develop cryobanking services for the aquaculture industry in Australia.

Initial capital investments

In this study, initial capital investments were divided into essential and optional equipment, listed in Table 2. Equipment was categorised based on a paper by Caffey & Tiersh (2000) that analysed the costs for integrating a basic cryopreservation program into an existing fish hatchery. The only exceptions were the compound microscope which has been listed as essential in the current study as it is required to assess sperm quality, a critical step in sperm cryopreservation for selective breeding programs and exclusion of dewars for sperm shipment because it was assumed in this study that the breeding nucleus would be held at one locality and the distribution of genetic gains would be achieved through selling progenies produced from genetically improved broodstock only. A miscellaneous cost of \$4000 was also added to cover the specific requirements in different species groups, such as UV spawning induction system in the abalone group.

The initial capital investment was estimated to be \$71,416, with approximately 45% (\$32,212) of costs for essential equipment and 55% (\$39,204) for optional investments (Table 2). However, as research and development is required to further refine current techniques and protocols across all four species groups considered, it is likely that both essential and optional equipment will be needed in the provision of a desired cryobanking service. Therefore, the total investment listed in this study would be more realistic although both essential and total investments have been used in the subsequent analyses.

Table 2. Capital costs for establishing a sperm cryobanking service at a hatchery involved in a selective breeding program

Item	Unit no	Unit price	Subtotal
A. Essential equipment			
Liquid nitrogen storage dewar (35 L)	3	1663.20	4989.60
Roller base for liquid nitrogen storage dewar	2	321.00	642.00
Liquid nitrogen transfer hose & phase separator	1	393.40	393.40
Liquid nitrogen level measuring rod	1	8.40	8.40
Thermometer (-200 to 70°C)	1	298.50	298.50
Analytical balance (0.01g precision)	1	3066.00	3066.00
Spatulas	2	21.43	42.86
Compound microscope	1	5386.50	5386.50
Cryo-gloves (pairs)	1	328.38	328.50
Safety goggles (pairs)	1	18.38	18.38
Laboratory timer	2	59.33	118.66
100 mL measuring cylinder	1	37.50	37.50
250 mL measuring cylinder	1	76.50	76.50
Stir plate	1	772.00	772.00
Stir bars	5	18.38	91.90
20 L carboy	1	193.20	193.20
Straight forcept	1	53.55	53.55
Curved forcept	1	54.08	54.08
Big autoclave tray	1	111.30	111.30
Test tube racks	2	57.75	115.50
Glassware		1000.00	1000.00
Pipette pumps	2	45.68	91.36
Heavy duty scissors	1	33.00	33.00
Miscellaneous		4000.00	4000.00
Loan interest over 5 years	8%	10289.00	10289.00
<i>Total essential equipment investment</i>			32211.84
B. Optional equipment			
Pipettor (10-100 µL)	1	499.80	499.80
Pipettor (100-1000 µL)	1	499.80	499.80
Pipettor (1000-5000 µL)	1	499.80	499.80
Low-level alarms (for storage dewars)	2	564.90	564.90
Water bath	1	1790.25	1790.25
Vapour pressure osmometer	1	6952.00	6952.00
Programmable freezing machine*	1	13310.00	13310.00
Others		2000.00	2000.00
Loan interest over 5 years	8%	12522.35	12522.35
<i>Total optional equipment investment</i>			39203.80
Total equipment investment (optional + essential)			71415.65

* Prices from Cryologic, Australia, 2010.

Costs analysis on freezing materials

In this study, “family unit” is defined as sufficient quantity of sperm to produce one full-sib family in the selective breeding program following cryopreservation. The “family unit” costs were dictated by the capacity of goblet (Five 0.5mL straws per goblet) and the need to store straws from different males in different goblets in order to avoid cross contaminations and any potential damage to straws of other individuals during the thawing processes (if straws from more than one individual are mixed in one goblet).

The “family unit” costs or per male costs of freezing materials, straws or cryovial, goblets and canes range from \$1.69 to \$4.87 depending on the species (Table 3). These costs would be halved if back-ups are not needed. As shown in Table 3 the number of straws or cryovials required per male ranges from 1 to 5, which was calculated according to the fertilisation and survival rates estimated at critical developmental stages listed in the table. Although these estimations are conservative, further reductions in rates would have limited impact on freezing materials costs as the goblet is not being used to capacity in all species groups (except finfish). The biological parameters considered in other groups is not relevant in the prawn group as only two spermatophores will be cryopreserved from an individual male.

Compared with other costs associated with sperm cryopreservation, the costs for freezing materials per male are relative small. However, the number of males cryopreserved is critical in determining the number of canisters and dewars, impacting the operating and annual maintenance costs listed in the subsequent analyses.

Table 3. Biological parameters and freezing materials used to calculate costs per male per generation in different species groups

Item	Species group			
	Finfish (salmon)	Prawn (tiger)	Bivalve (Pacific oyster)	Abalone (greenlip)
A. Biological parameters				
Number of individuals per family required for performance evaluation	50 ¹		1200 ²	100 ³
Fertilisation rate with cryopreserved sperm	90.0%		90.0%	90.0%
Hatching or D-larval rate	80.0%		35.0%	25.0%
Survival rate from previous stages to day 25-30 post-fertilisation or metamorphosed	12.5%		50.0%	60.0%
Survival rate from previous stage to tagging or start of grow-out assessment	50.0%		90.0%	5.0%
Minimum number of eggs required at the fertilisation	1111		8466	14815
Number of eggs that can be fertilised by one 0.5 mL straw	225 ⁴		31250 ⁵	12500 ⁶
B. Freezing material				
Number of straws or cryo-vials required per male	5	1	1	2
Straw or cryovial capacity (0.5 mL straw or 2.0 mL cryo-vial)	0.5	2.0	0.5	0.5
Unit price	0.17	0.39	0.17	0.17
Straw or cryovial cost per male	0.85	0.39	0.17	0.34
Number of goblets required per male	1		1	1
Unit price	0.44		0.44	0.44
Goblet cost per male	0.44		0.44	0.44
Number of canes required per male	0.5	0.2	0.5	0.5
Unit price	2.29	2.29	2.29	2.29
Cane cost per male	1.14	0.46	1.14	1.14
Total freezing material costs per male (family unit)	2.43	0.85	1.75	1.92
Total freezing material costs per male with a backup	4.87	1.69	3.51	3.85

¹. CSIRO. ². Ryan *et al* 2006. ³. Li 2008. ⁴. Jodun *et al* 2006. ⁵. Li 2009. ⁶. Li 2004.

Cost analysis on cryopreservation and annual maintenance

The data provided in Table 4 is based on a breeding nucleus size of 100 full-sib families across all four species groups, allowing the relative costs among these groups to be compared. Changes in cryopreservation and annual maintenance costs with nucleus population sizes from 50 to 400 families per generation are presented in Figure 2. It should be noted that the total generation costs per family unit (male) will also be determined by the generation interval anticipated for each species group. For example, the generation costs per family unit for abalone would be cryopreservation costs plus three times the annual maintenance costs. The generation costs per family unit are not presented in this study because they are not required in the subsequent analyses.

The number of male broodstock per freezing run is dictated by two factors: a) the capacity of the liquid nitrogen vapour freezing device (thirty 0.5 mL straws or 2.0 mL cryovials in this analysis); and b) the maximum number of males that can be processed each run without compromising sperm quality (5 males in this study). Finfish groups are mainly determined by the first factor, while other species groups are mainly dictated by the second factor.

A breeding nucleus of 100 full-sib families per generation has estimated cryopreservation costs per family unit per generation of \$60.06, \$53.67, \$54.91 and \$141.67 for finfish, prawn, bivalve and abalone groups, respectively, based on an initial capital investment that includes essential equipment only. These costs increase to \$61.21, \$54.81, \$56.05 and \$145.10 respectively, if initial capital investment includes both essential and optional equipment. However, the costs increases are marginal, with a difference of less than \$4.00 in each case (Table 4). The annual maintenance costs per family unit are, on the other hand, the same across species groups, being \$89.38 if the initial capital investment includes essential equipment only or \$128.58 if both essential and optional equipment is included. These costs equate to \$0.24 or \$0.35 (essential and optional equipment) per day per family unit.

The cryopreservation costs per family unit remain constant with changes in the breeding nucleus size from 50 to 400 full-sib families (Figure 2). The costs in abalone

group (spawned naturally, about \$145/family unit) are about three times higher than those in other groups (strip-spawned, about \$55/family unit). This is not surprising as the time period required to collect sperm from the desired number of males would be more than three times longer in abalone than that in the groups that can be strip-spawned. The annual maintenance costs per male, on the other hand, decrease with an increase in the breeding nucleus size from 50 to 400 full-sib families per generation. An increase in family numbers from 50 to 150, substantially reduces the costs from approximately \$260 or \$180 per family unit per year to \$86 or \$60 per family unit per year, depending on whether both essential and optional equipment or essential equipment only has been included in the assessment of the initial capital investment. Both costs decrease slowly with a further increase in the family numbers per generation to approximately \$37 and \$26 at 350 families in the groups where their sperm have been stored in 0.5 mL straws (finfish, bivalve and abalone groups; Figure 2A, C, D). This is dependent on the number of canes that can be held per storage dewar, with 180 being standard in a 35L dewar. Each storage dewar could therefore accommodate sperm from a maximum of 360 family units according to biological parameters used in this study. However, when the family number increases to 400, the annual costs only increase slightly to \$48 (optional & essential equipment) and \$38 (essential only) per family unit, although an additional dewar is added. These costs will be reduced with a further increase in family numbers per generation (data not show).

The highest number of males required per generation in the breeding programs surveyed in this project was 300 in Sydney rock oysters (7.4. Breeding programs in Australian aquaculture industry and their short- and long-term requirements for cryobanking services). Even in this instance there would be at least 60 goblets or 300 straws available for other purposes such as the storage of sperm for the establishment of cross generation controls, although these are not included in the analysis. It is anticipated that the additional costs associated with achieving this would come from the freezing materials (straws, goblets and canes), which are minimal, as the activities would be combined with the core activities and no extra costs are required for annual maintenance.

Table 4. Operating costs for gamete cryopreservation

Item	Unit size	Unit price	Species group			
			Finfish (salmon)	Prawn (tiger)	Bivalve (Pacific oyster)	Abalone (Greenlip)
A. Basic biological and operational parameters used in the calculation						
Number of families per generation			100	100	100	100
Generation cycle			3	1	2	3
Number of male broodstock can be managed per freezing run			3	5	5	5
Litres of liquid nitrogen required per run			3	3	3	3
B. Cryopreservation costs						
Liquid nitrogen	1 L	3.47 ³	353.94	235.96	235.96	235.96
10 years straight line equipment depreciation (E) ¹			89.48	89.48	89.48	268.43
10 years straight line equipment depreciation (E + O) ²			198.38	198.38	198.38	595.13
Technicians (2x10% for 100 abalone families or 300 others)	FTE	60000.00	4000.00	4000.00	4000.00	1200.00
Freezing material costs per generation			486.90	169.16	350.90	384.90
Cleaning materials			30.00	30.00	30.00	30.00
Distilled (Milli-Q) water		20.00	20.00	20.00		
NaCl	500g	76.65	38.33	19.16		
KCl	500g	116.55	58.28	29.14		
CaCl ₂ ·2H ₂ O	500g	79.28				
MgSO ₄	500g	122.85		30.71		
Na ₂ HPO ₄	500g	148.05		37.01		
KH ₂ PO ₄	500g	100.80		25.20		
Na ₂ HCO ₃	500g	48.30		12.08		
Glucose	500g	35.70	17.85	8.93		
Dimethyl sulphoxide (DMSO)	500mL	217.35	108.68	54.34	72.45	108.68
Trehalose	100g	348.60			116.20	
0.2 micron filter	each	80.00				80.00
Centrifuge tubes (15mL)	500/pk	159.00	31.80	31.80	31.80	31.80
Tris-Trisma base	100g	43.05	21.53			

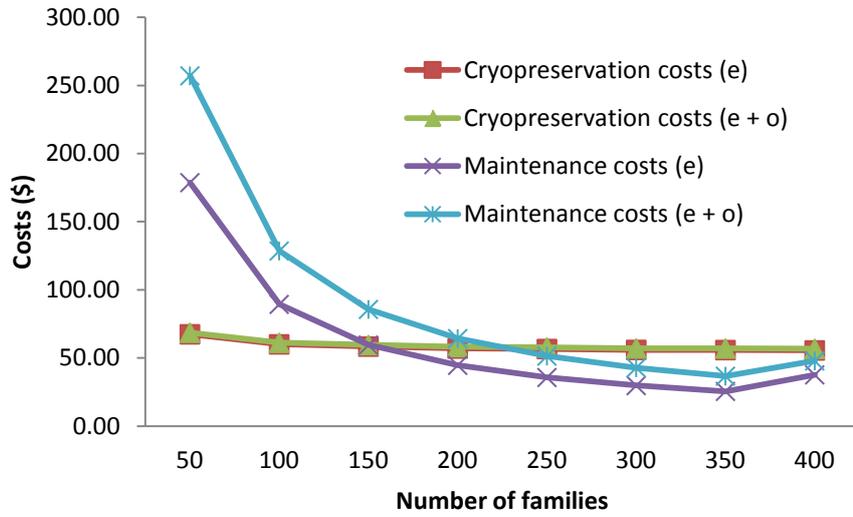
Glycine	100g	56.18	28.09			
Chicken eggs	1 dozen	3.45	3.45			
Polyvinylalcohol (PVA) sealing powder	25g	135.45	67.73	33.86	45.15	67.73
Bags of ice cubes (one bag per day)	1bag	2.5	50.00	50.00	37.50	50.00
Latex gloves	50 pairs/pk	24.15	96.60	96.60	96.60	96.60
HEPES	25g	79.80	39.90			
Microscope slides	72/pk	34.13	52.14	52.14	52.14	52.14
1mL disposable pippets	800/pk	205.80	25.73	25.73	25.73	25.73
10mL disposable pippets	100/pk	301.35	60.27	60.27	45.20	60.27
Contingency (5% of cryopreservation costs)			286.02	255.58	261.46	674.61
Cryopreservation costs per generation (E)¹			6006.33	5367.14	5490.56	14166.84
Cryopreservation costs per generation (E+O)²			6120.67	5481.48	5604.91	14509.87
Cryopreservation costs per family unit per generation (E)¹			60.06	35.67	54.91	141.67
Cryopreservation costs per family unit per generation (E+O)²			61.21	54.81	56.05	145.10
C. Annual maintenance costs						
10 years straight line equipment depreciation (E) ¹	12 months		3221.18	3221.18	3221.18	3221.18
10 years straight line equipment depreciation (E+O) ²	12 months		7141.56	7141.56	7141.56	7141.56
Liquid nitrogen	1 litre	3.47	2498.40	2498.40	2498.40	2498.40
5% FTE technician	FTE	60000.00	3000.00	3000.00	3000.00	3000.00
Contingency (2.5% annual maintenance costs specified)			217.99	217.99	217.99	217.99
Total annual maintenance costs per family unit (E)¹			89.38	89.38	89.38	89.38
Total annual maintenance costs per family unit (E+O)²			128.58	128.58	128.58	128.58
Daily maintenance costs per family unit (E)¹			0.24	0.24	0.24	0.24
Daily maintenance costs per family unit (E+O)²			0.35	0.35	0.35	0.35

¹ Only costs for essential equipment are included.

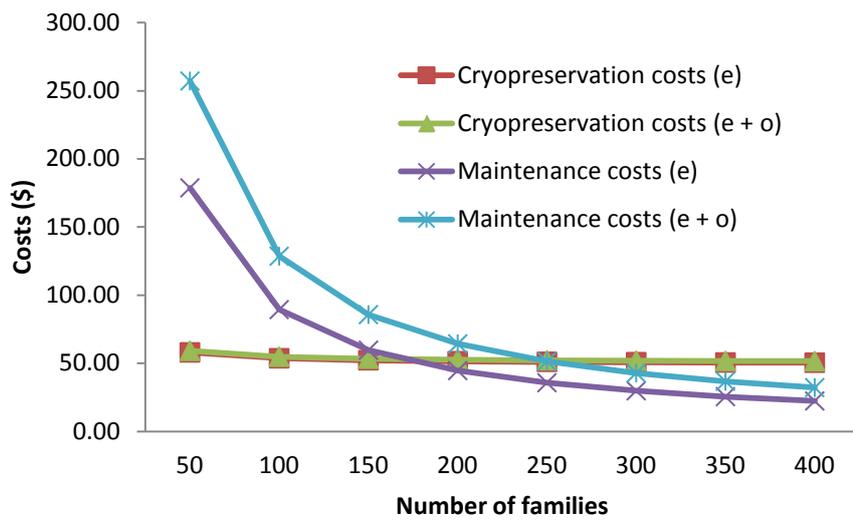
² Costs for both essential and optional equipment are included.

³ Price at Adelaide depot of BOC limited in July 2010.

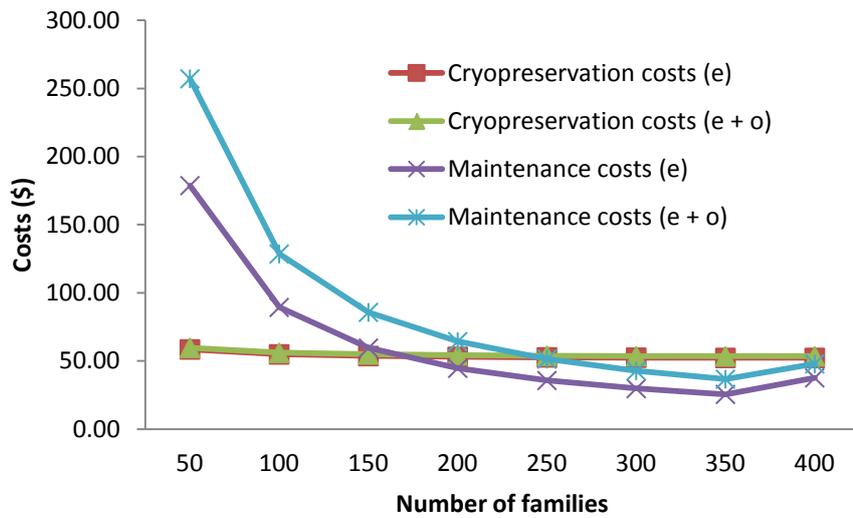
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B.



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D.

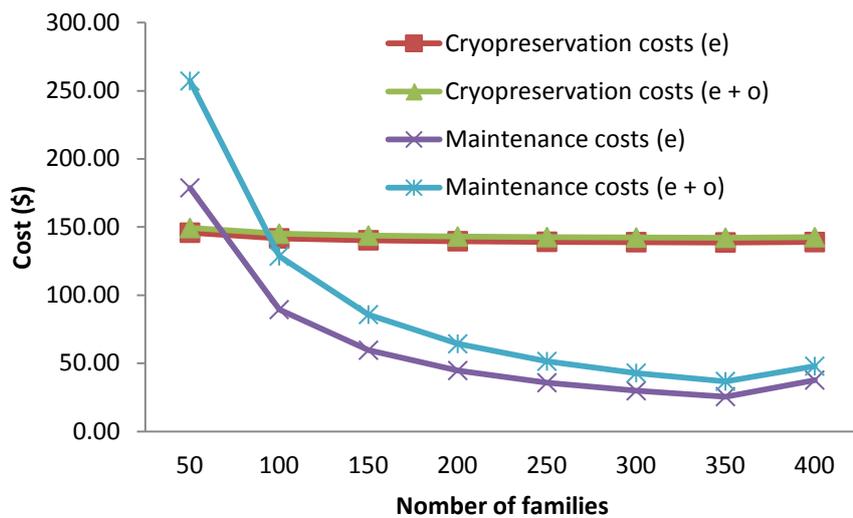


Figure 2. Changes in cryopreservation costs and annual maintenance costs with an increase in the number of families per generation/year class in each species group. A. Finfish group; B. Prawn group; C. Bivalve group, and D. Abalone group. e and o represent essential and optional equipment costs, respectively.

The annual maintenance cost, converted to a daily rate per family, decreases across all species groups as the number of families increases, ranging from \$0.7 to \$0.07 depending on the species, number of families and whether essential and optional equipment or just optional equipment is included in the estimation (Figure 3).

It should note that in the above analyses the costs for infrastructure and shipment of cryopreserved sperm between hatcheries and off-site cryopreservation storage facilities (if needed) are not included.

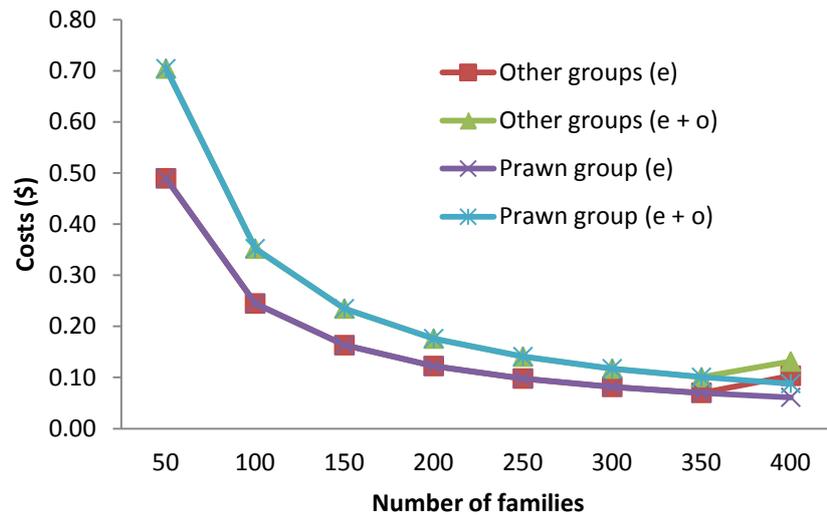


Figure 3. Changes in daily maintenance costs with an increase in the number of families per generation/year class in prawns and other species groups (finfish, bivalve and abalone groups). e and o represent essential and optional equipment costs, respectively.

Although the analysis on integrating a cryobanking service into a commercial hatchery production has not been conducted in this study, the cost model developed could be used to estimate the production capacity. Theoretically, up to 607,500, 84,375,000 and 3,375,000 fertilised eggs can be produced per dewar for finfish, bivalve and abalone groups respectively, according to the biological parameters used in this study and the maximum number of 0.5 mL straws that can be stored in a 35L storage dewar (3000 straws/dewar, Caffey & Tiersch 2000).

7.4. Concluding remarks

The cost analyses reveal that:

- The total initial capital investment is approximately \$71,416 with 45% (\$32,212) of the costs for essential equipment and 55% (\$39,204) allocated for optional equipment.
- The “family unit” or per male costs for freezing materials (straws/cryovials, goblets and canes) are \$2.43, \$0.85, \$1.75 and \$1.92 for finfish, prawn, bivalve and abalone groups, respectively, based on the biological parameters and the storage system configurations defined in this study,.
- Sperm from a maximum of 360 males can be stored per dewar according to the storage capacity of a 35L storage dewar defined in this study.
- The cryopreservation costs per family unit (per male) remain consistent with changes in the breeding nucleus size from 50 to 400 full-sib families (Figure 2) in each species group estimated. Costs are approximately three times higher in groups where sperm is collected through natural spawning such as abalone (about \$145/family unit) in comparison to species that are strip-spawning such as salmon, prawns and oysters (about \$55/family unit).
- The annual maintenance costs per family unit (per male) decreases with an increase in the breeding nucleus size from 50 to 400 full-sib families per generation. The costs reduce substantially from approximately \$260 to \$86 per male per year with an increase in the family numbers from 50 to 150. The costs then decrease slowly with further increases in family numbers to \$37 at 350 families. The annual maintenance costs can be converted into daily maintenance costs, resulting in \$0.70, \$0.35, \$0.24 and \$0.1 per male per day at the breeding nucleus sizes of 50, 100, 150 and 350 families, respectively.
- Up to 607,500, 84,375,000 and 3,375,000 fertilised eggs can theoretically be produced by the 0.5mL straws stored in a 35L dewar in finfish, bivalve and abalone groups, respectively, according to the biological parameters and cryostorage equipment configurations used in this study.

8. SHORT- AND LONG-TERM REQUIREMENTS FOR CRYOBANKING SERVICES IN AUSTRALIAN AQUACULTURE INDUSTRY AND KEY CHARACTERS OF CRYOPRESERVATION SERVICE FACILITIES INVESTIGATED

8.1. Selective breeding programs in Australian aquaculture industry and their short- and long-term requirements for cryobanking services

In Australia, selective breeding programs have been established for Atlantic salmon, Pacific oyster, Sydney rock oyster, abalone and prawn aquaculture industries and are expected to soon be developed in the barramundi and pearl oyster industries. All of these breeding programs use, or will soon be using, the family based approach; producing families each generation for evaluation and selection.

To assist the project in understanding the short- and long-term requirements for a proposed cryobanking service for the different species investigated in this project, an industry survey was conducted. The survey provided industry members with background information about the project and a survey form (Appendix 3), with follow up phone or email communication for clarifications if needed. Key outcomes from the industry survey can be summarised as follows:

- a. The proposed cryobanking service should assist both commercial hatchery production and selective breeding programs in all industry sectors surveyed, noting that this project focused on the selective breeding programs only.
- b. All selective breeding programs expected their replacement strategy to be generation by generation.
- c. Maintaining genetic diversities through cryobanking have not been considered a priority at this stage of breeding program development.
- d. The short- and long-term requirements for numbers of males and female broodstock within the program are the same, ranging from 100 individuals per sex in Pacific oysters to 300 in Sydney rock oysters.
- e. All the breeding programs expect ownership of their germplasms stored off-site.
- f. The breeding programs have limited, if any, equipment required for cryopreservation and cryostorage.

- g. All programs, except for Pacific oyster, indicated they have trained staff who can conduct cryopreservation on-site.
- h. No specific cryopreservation data management system exists across any of the breeding programs.
- i. Sperm quality is evaluated in the industry by visual motility assessment and fertility examination.
- j. Sperm cryopreservation protocols exist in all species surveyed, while oocyte cryopreservation protocols were published for Pacific oysters only.

8.2. Key characters of cryopreservation service facilities investigated

Cryobanking facilities that are used in livestock research and/or improvement programs have also been investigated as part of this study, including visits to SARDI livestock cryobanking facility at Turretfield SA, Brecon Breeders and a commercial livestock cryobanking company at Keith SA, as well as discussions with staff at Australian Phenomics Facility at The Australian National University, ACT and Cryogenetics in Norway. However, requests to visit cryobanking facilities for assisting human reproduction were declined.

The three Australian organisations investigated in this study represent three types of operational/business models: 1) full commercial business model (Brecon Breeders); 2) public fund subsidised business model (Australian Phenomics Facility); and 3) research supported operational model (SARDI livestock cryobanking facility).

The Australian Phenomics Facility (APF) is financially supported by the Australian Government through NHMRC, NCRIS and EIF. The APF is a non-profit repository of mouse strains used in medical research. At APF, the Scientific Advisory and Access Committee of the Australian Phenomics Network decides which mouse strains are stored at the facility. The facility uses two different cost models for the storage of sperm:

- A) Strains for distribution to other researchers within Australia costs \$350/strain (10 straws per male plus 1 straw for quality test and 5 males per strain). However, the

depositor needs to pay for 1) shipping and maintenance of animals and 2) quality control (same as for cost model B).

B) Strains not freely distributable are costed at the following rates:

- Cryopreservation
\$750 per strain (5 males)
- Quality control
\$60 per strain (5 males) - subjective microscopy analysis.
\$800 per strain - performance of IVF + embryo transfer
- Annual maintenance
\$100 per strain

Plus costs for shipping and maintenance of animals.

In both cost models, a charge of \$400 is applied for each purchase of three straws from APF. Sperm cryopreservation is conducted on-site at APF and the parameters used for quality control are: sperm concentration, motility, forward motility and gross physical characteristics such as damage to sperm head or tail. These quality control measures are critical in some animals such as stallions where sperm from about 30% of males does not survive the freezing process for artificial insemination.

The cost models used in private cryopreservation companies (full commercial business model) vary widely and are mainly based on the nature/activities of the service required. They provide a range of services from gametes and embryo cryopreservation, artificial insemination, to provision of expert advice on the use of artificial breeding to increase the rate of genetic improvement. With regard to sperm cryopreservation, the three activities (cryopreservation, quality control and annual maintenance) described for the public fund subsidised business model can be conducted and charged respectively according to customers requirements. The costs for the first two components are comparable to those used at APF for stains that are not freely distributable. However, in some cases the annual maintenance cost can be up to as much as about \$1000 to store a quantity of sperm solution equivalent to five 0.5 mL straws (one family unit), according to estimates in this study. It should be noted the full commercial model would also include infrastructure depreciation and administration costs.

Costs for research projects supported operational model are very hard to specify as the cryopreservation service is mainly required and funded by the R&D projects and the research organisation hosting the facilities.

The common features of these facilities are:

- The activities required for cryopreservation are undertaken by trained and skilful staff.
- Scientifically proven protocols are in place for the species and material processed.
- Rules for sanitary/veterinary requirements are clear.
- Both manual and automatic alarm systems are used to monitor the liquid nitrogen in the storage containers (such as storage dewars).
- All these facilities use 35L dewars for long-term storage.
- Facilities located at public organisations have a R&D team to improve cryopreservation techniques.
- Web based data management system is under development at APF.
- At Brecon Breeders, depositors are responsible for insuring cryopreserved materials stored at their facility.

Cryogenetics is a commercial entity in Norway, specialising in Atlantic salmon, cod and rainbow trout milt cryopreservation, storage, distribution, thawing and fertilisation. The protocols used at this facility have been developed or evaluated by the company. Key features of their business/operational model are:

- They have a R&D team to develop and optimise protocols for new species and/or new materials.
- They provide a cryoservice in Norway and Northern European countries, with customers shipping milt to their laboratory for cryopreservation and storage, although their method could be operated by a trained technician on a farm.
- Milt stored at their facility is customers' property.
- They offer insurance for the materials stored at their facility (up to US\$100,000 per canister).

- Costs of their cryobanking services are determined on a case by case basis according to the number of units processed per season and a monthly storage charge.

Cryogenetics is interested in expanding their business overseas and has set up a similar system in Canada and Chile.

9. POTENTIAL OPTIONS TO ESTABLISH CRYOBANKING SERVICE FOR AQUACULTURE BREEDING PROGRAMS IN AUSTRALIA

There are a few potential options available for establishing a cryobanking service for aquaculture breeding programs in Australia, including:

1. Establishing cryobanking service on the farm(s) participating in the selective breeding program.
2. Establishing the service at an existing livestock cryobanking facility.
3. Establishing the service at a public fund subsidised facility.
4. Establishing the service at a research organisation.
5. Establishing a new cryobanking centre.
6. Establishing the service by a stepwise approach in conjunction with a research or an existing cryobanking facility.

All aquaculture sectors survey in this study indicated that they would like to use a cryobanking service for both commercial hatchery production and genetic improvement programs. The analysis in the previous section shows that a maximum of two storage dewars (one each for primary and back up storage) would be needed for each selective breeding program according to the family numbers predicted for their long-term requirement.

At this stage it is very hard to predict the cryo-storage capacities needed for each species to assist commercial hatchery productions as this technique has not yet been applied in Australian aquaculture industries for various reasons. In this study they are therefore estimated according to the potential industry adoption levels (at a 25% interval) for each aquaculture species (Table 5). The total production for each species is based on the data published by ABARE (2009) or the Pearling Industry Advisory Committee (2005). It is hoped that the number of dewars required at each industry adaptation levels would provide some indication of the potential business scope of the proposed cryobanking service to the Australian aquaculture industry.

Table 5. Annual production and number of 35L storage dewars required for sperm/spermatophore cryopreservation at different industry adoption levels (25% interval)¹

Annual production or adoption level	Number of 35L dewars needed at different industry adoption levels						
	Salmon	Barramundi	Prawn	Abalone	Pearl oysters	Sydney rock oysters	Pacific oysters
Annual production	24248 (ton)	3361 (ton)	3088 (ton)	504 (ton)	1342 (unit) [#]	4500 (ton)	7960 (ton)
Adoption level							
100%	247	171	2	23	1	8	12
75%	186	129	2	18	1	6	9
50%	124	86	1	12	1	4	6
25%	62	43	1	6	1	2	3

¹ It is assumed that all required sperm in each species will be cryopreserved and applied in the commercial hatchery production once a year.

* Data for some species such Sydney rock oysters would be slightly higher than their real production in the 2008/09 financial year as the report has put the productions from both Sydney rock and Pacific oysters together.

[#] Pearling Industry Advisory Committee 2005. One unit equals to 1000 pearl oysters.

As showed in Table 5, the number of dewars required for assisting commercial hatchery production is highly variable among species sectors. This is mainly due to two factors: a) the number of eggs that can be fertilised by a standard volume of cryopreserved sperm; and b) the number of fertilised eggs that are needed per year or spawning season. For example, using existing protocols a 0.5mL straw fertilises 31,250 Pacific oyster eggs, the same amount in salmon only fertilises 225 eggs, with salmon needing nearly 140 times more straws to fertilise the same number of eggs. Unfortunately, the amount of fertilised eggs required in salmon is only 7 times less than in Pacific oysters according to the biological parameters used in this study. However, this does not mean that the implementation of sperm cryopreservation to assist commercial hatchery production in Pacific oysters would be more profitable than in salmon. To establish this, a detailed cost benefit analysis based on a clear integration strategy that is species and farming system specific would be needed and is beyond the scope of this study. However, it is clear that the volume of cryopreserved straws required for hatchery application in finfish species would be challenging. If this is to be considered, further improvements in cryopreservation techniques with larger freezing containers such as cryo-bags and freezing equipment that could handle larger amount of sperm per freezing run would be one of the research priorities. Establishing cryobanking facilities for hatchery production of shellfish species is likely to be even more challenging. In addition to the lack of information on cost benefit analysis with regard to the integration of sperm cryopreservation techniques into hatchery productions, the potential cryobanking business scope in these industries is small and cannot warrant the establishment of an independent cryobanking facility at the current production level. Establishment of a national centre would also be difficult due to issues related to funding, translation, diseases, existing business opportunities, etc. Determining the location of a proposed centre would also be hard unless the funding for the cryobanking facility came from an independent investor or a public agent, as transportation of cryopreserved sperm can be costly, especially with the amount of straws involved in finfish species. All these suggest that a stepwise approach might be a logical option, starting with the development of cryobanking capacity to service the needs of selective breeding programs.

The drivers behind establishing a cryobanking service for assisting genetic improvement programs differ from that of commercial hatchery productions. Breeding programs are primarily interested in quality measures such as preservation of genetic diversity to meet current and future consumer demands; implementation of desired mating design; and minimisation of potential impacts from catastrophes and disease outbreaks, etc.

The two main costs: the cryopreservation cost and the annual or daily maintenance cost for establishing an on farm cryobanking service to assist selective breeding programs has been analysed in previous sections. They have also been compared with the three existing business/operation models currently used in the cryobanking facilities investigated. These costs are similar across the three business model evaluated and a decision is therefore likely to be based on the most practical option that meets the breeding programs needs.

➤ Initial investment

A substantial initial investment would be needed if the cryobanking service was to be established on farms or as a new centre. In addition, it is anticipated that over the first few years the initial demand for a cryobanking service would not be large enough to support the establishment of a new centre unless high service fees were imposed or other businesses were incorporated.

➤ Provision of R&D

Existing cryopreservation protocols have not been assessed or optimised according to the equipment available and the hatchery production systems used by the selective breeding programs in Australia. The establishment of a standard protocol to suit local conditions would need to be a priority if the desired cryobanking service was to be achieved. This should be done on the farm where cryopreservation is to occur.

With regard to addressing the technical gaps listed in the next section, it is suggested that required studies be conducted at R&D organisations that have the research capability and specialised equipment in the field of cryopreservation, supported by experts in related science fields.

➤ Cryopreservation

For those species that can be strip spawned cryopreservation can be conducted on farm or off-site at other organisations, as long as fresh sperm can be transported without affecting its quality. Cryopreservation must be conducted on farm or at off-site facilities where natural spawning can be managed, for species that cannot be strip spawned.

Experience overseas suggest that the aquaculture industry prefer trained staff from cryobanking facilities to provide the cryobanking service, even on-site as the procedure is very sensitive to variations.

➤ Storage of cryopreserved gametes

Two key factors need to be considered in the storage of cryopreserved gametes: a) natural disasters; and b) cross contamination. The first issue can be avoided to some extent by storing cryopreserved materials at two different sites. From an economic point of view, one could be on-farm, the other on an off-site facility such as a research organisation, cryobanking facilities, etc. The second issue can be addressed to a certain extent by storing materials from one farm in their own cryostorage device. This means that if a livestock cryobanking facility is used, the cryopreserved materials from aquatic species should not be mixed with the straws and cryo-vials from livestock. Therefore, dedicated storage dewars or other cryostorage devices would be required for materials for different farms and different species.

➤ Quality control measures

At the moment the most reliable method of assessing the quality of cryopreserved gametes (sperm) in all species investigated in this study is by calculating the fertilisation rate. Other parameters evaluated so far cannot ensure the quantity of sperm cryopreserved will be enough for the proposed purpose. This means that at this stage cryopreservation is best conducted either on farm or at a research facility so that the quality of cryopreserved sperm can be checked at the required time, unless a proven cryopreservation protocol has been established.

➤ Ownership of cryopreserved materials stored

The industry survey indicates that the depositor wants to maintain ownership of their cryopreserved materials deposited. This could be properly managed in all options considered in this study.

➤ Translocation

Each state in Australia has its own translocation and disease management policies and codes of practice. These might have some implications on cryopreservation practices, especially when gametes are to be moved between states. Further investigation would be needed if this kind of practice was likely to occur.

After reviewing the current status of cryopreservation in Australia this project recommends a stepwise approach in conjunction with a research organisation or an existing cryobanking facility, as the most practical option to establish a cryobanking service for aquaculture selective breeding programs in Australia. This approach would not only meet current demand but also effectively address the key technical gaps and concerns. Key steps in this process include:

- A) Establishing a project to secure the equipment listed in Table 2, noting that some equipment can be rented/provided by potential research providers or commercial suppliers.
- B) Optimisation and standardisation of published protocols/methods using equipment and hatchery production systems currently available to breeding programs. This is critical in determining the scope of facilities required to achieve the cryobanking service for breeding programs. This should either be conducted by or at least supervised by experts in the fields of cryopreservation and reproduction biology of the target species.
- C) Establishment of a training program to improve procedural efficiency and personnel recruitment.
- D) Establishment of a contract with an existing livestock cryobanking facility or a research organisation.

Theoretically, all three components involved in a cryobanking service (cryopreservation, storage, and quality assessment) can be contracted out separately or together depending on which arrangement would best suit the breeding program and cryopreservation technical requirements.

E) R&D is required to address the technical gaps listed in section 10.

As discussed in the next section, the proposed cryobanking service would provide a comprehensive service to selective breeding programs, such as desired mating, reconstruction of the breeding nucleus when needed, etc. However, reconstruction of breeding nucleus cannot be achieved by cryopreservation of sperm only. Most gaps listed in section 10 are generic to all species. It would be better if they could collectively be funded by all breeding programs and their respective growers associations.

F) Review of the long-term requirement of selective breeding programs

As aquaculture breeding programs develop it is likely that the genetic diversity of the breeding nucleus will decline due to the natural tendency of breeding programs to select individuals of high economic merit with high levels of accuracy, and the limited number of broodstock that can be held in a facility. As a consequence maintaining genetic diversity to meet current and future demands is likely to become a critical issue. This should be taken into consideration when establishing the long-term storage capabilities of a proposed cryobanking service.

An alternative approach is to work with most fishery and aquaculture sectors to secure a fund to establish a cryobanking service similar to the Australian Phenomics Facility, with its main aim being to conserve the genetic diversity of marine biological resources. Most aquatic cryobanking facilities in the world have been established using this kind of funding support.

10. TECHNICAL GAPS AND/OR IMPROVEMENTS IDENTIFIED FOR ESTABLISHING A CRYOBANKING SERVICE IN AQUACULTURE BREEDING PROGRAMS IN AUSTRALIA

To achieve a cryobanking service for selective breeding programs in Australia it is necessary to ensure that:

1. Sperm cryopreserved from each individual male is enough to produce the number of progenies required in the breeding program; and
2. the breeding nucleus can be reconstructed when needed. The nucleus cannot be established from sperm only unless female broodstock can be produced through androgenesis or a combination of sex reversal techniques, depending on the gender determination mechanism in the species of interest. However, production of androgens (progenies with chromosomes from males only) is challenging and has not been evaluated for most fish and shellfish species. Even if these techniques are available to the species of interest, the whole genome cannot be recovered with sperm only as cytoplasmic effects will be lost or altered (Gandini *et al* 2007). In addition, the genetic variation in sperm can only represent those components from male broodstock; the unique components from females will be lost. Crosses with females from wild or unselected stocks will erode the genetic gains that have been achieved.

To overcome the two key concerns above, or issues specific to the provision of a cryobanking service to selective breeding programs, the following gaps and/or critical improvements are identified and need to be addressed as a priority:

- Optimisation of existing protocols to suit local conditions (cryopreservation equipment and hatchery production systems)

The equipment used in published researches and protocols has not been standardised and is sometimes self constructed. The sensitivity to variation in each step of cryopreservation means that protocols need to be optimised according to the equipment and systems available to the breeding program.

- Temporal variation in gamete qualities
Unpublished data on abalone indicates that sperm collected toward the end of the spawning period is more sensitive to cryoprotectant treatments than those collected mid way through the spawning period, although both can produce similar fertilisation rate in controls. Information on other species is lacking. If this kind of sperm is used, a larger amount of sperm per male would be needed for cryopreservation so that the number of progenies required in the breeding program can be produced.

- Individual variation in sperm “freezability”
Difference in sperm “freezability” among males has contributed to variations in fertility rates among full-sib crosses in Pacific oysters (Adams *et al* 2008). A similar phenomenon has also been found in mammals (Massip *et al* 2004). Factors causing variations in sperm “freezability” are not well understood in the aquatic species studied to date and cannot be avoided. Therefore, strategies that could minimise its potential impact on provision of a cryobanking service to selective breeding programs need to be developed.

- Oocyte/embryo cryopreservation
The oocyte/embryo cryopreservation techniques published for other species, except in Pacific oysters (Tervit *et al* 2005), are not mature enough to be used in a cryobanking service for selective breeding programs. Establishing these techniques is critical in being able to reconstruct the breeding nucleus however; developing these techniques is proving challenging.

- Performance of progenies from gametes that have both been cryopreserved
No information is available on the performance of progenies that have resulted from gametes that have both been cryopreserved although the benefits are obvious as cryopreserved sperm and oocytes can be used to reconstruct the breeding nucleus.

Improvement in the following techniques would further enhance the cryobanking service qualities to the breeding programs:

- **Non-destructive gamete collection**

The cryopreservation technique developed in bivalve species has been based on the destructive strip spawning method, sacrificing the selected broodstock (Lyons *et al* 2005, Acosta-Salmon *et al* 2007, Adams *et al* 2009, Dong *et al* 2009, Li 2009, Hui *et al* 2011). These very valuable genetic materials have then been wasted as these broodstock would have been selected from those animals that have the highest estimated genetic merits and should be used in commercialisation. Developing non-destructive techniques would allow genetic gains in the top performing broodstock to be retained for commercialisation.
- **Data management system**

Currently a comprehensive data management system to manage cryopreserved materials for aquaculture selective breeding programs is not available. If it was to be developed it should consider the requirements for both selective breeding programs and cryobanking services.
- **Development of alternative sperm quality assessment method**

Currently fertility assessment is the principal method to measure the post-thaw sperm qualities in all aquatic species, although other methods such as sperm motility, mitochondrial function, membrane integrity, etc have also been evaluated (Stoss & Holtz 1983, Adams *et al* 2003, Paniagua-Chavez *et al* 2006, Cabrita *et al* 2010). Published results indicate that sperm motility is a good indicator of the viability of fresh sperm; however, it is not always effective at predicting the fertilising ability in post-thaw sperm (Stoss & Holtz 1986, Paniagua-Chavez *et al* 2006). Other measures would normally require specialised equipment such as flow cytometry, which is not readily accessible in remote locations.
- **Infectious diseases and cross-contaminations**

Approximately 50 bacterial and 30 virus species have been isolated from diseased fish to date (Fryer & Bartholomew 1996). Some of them are highly infectious and can be transmitted horizontally and vertically (Fryer & Bartholomew 1996). It is therefore critical to consider and implement measures to prevent and control the

spread of diseases and viruses within a cryobanking service particularly as: a) disease screening to determine which gametes should be cryopreserved may be not only ineffective but also unrealistic (Thacker *et al* 1984); b) the occurrence of virus cross-contamination during liquid nitrogen storage and transportation of biological material and subsequent cross-infection of patients has been demonstrated previously (Clarke 1999, Bielanski *et al* 2003, Bielanski 2005a, b); and c) many marine bacteria can survive long-term cryostorage (Nimrat *et al* 2008).

If a cryobanking service was to be integrated into commercial hatchery production, a cost benefit analysis is strongly recommended to determine the optimum balance of investment strategies among cryobanking service, broodstock management, and short- and long-term production goals. As showed in Table 5, even the management of the number of cryocontainers required each spawning season is substantial and not a simple task, especially in finfish species. The analysis should include the assessment of (but not be limited to):

- potential future benefits (savings);
- operational costs under different broodstock management strategies;
- related initial investment strategies; and
- adoption strategies.

11. BENEFITS

This project is a scoping study to evaluate the potential of establishing a cryobanking service for aquaculture selective breeding programs in Australia. As such the expected potential benefits, as described in Appendix 3, will not be realised at this stage. This project however provides information that can be used to further progress the development of a cryobanking service, including:

1. The development of a model to estimate costs associated with cryobanking sufficient sperm, on-site, to establish a full-sib family in the selective breeding program, as well as estimating costs associated with changes to the size of the breeding nucleus (number of families) in each of the four species groups (finfish, prawn, bivalve and abalone groups) investigated in this study. This provides selective breeding programs with a basic tool to compare and select the cryobanking options that would best suit their short- and long-term needs.
2. The recommendation to consider a stepwise approach in developing a cryobanking service for the aquaculture breeding programs in Australia, involving a research or existing cryobanking facility that has been identified as a practical option for establishing this service. This kind of approach would not only meet the needs of those requiring cryobanking services, but also allows the issues and technical gaps identified in this study to be addressed. It should be noted that the option to establish a new independent facility should be considered if funding was available.
3. The identification of key technical gaps that need to be addressed through R&D in order to provide a comprehensive cryobanking service for aquaculture selective breeding programs.

12. FURTHER DEVELOPMENT

Cryobanking services have proven techniques in livestock, enhancing genetic improvement programs, achieving both genetic gains and distributing gains to the industry. However, these potential benefits have not been exploited in aquaculture selective breeding programs. This CRC project considered the options available for establishing cryobanking services in Australia, and recommends a stepwise approach involving a research or existing cryobanking facility. The project has also identified the key technical gaps and/or improvements that need to be addressed as a priority through R&D to effectively and efficiently deliver the proposed cryobanking service for aquaculture breeding programs in Australia.

13. PLANNED OUTCOMES

This project aimed at scoping the options available in establishing a cryobanking service for aquaculture selective breeding programs within the AS CRC. The planned outcomes for this project were achieved and include:

1. Cryopreservation and maintenance costs in provision of cryobanking service to aquaculture selective breeding programs

In this study, the key costs associated with establishing a cryobanking service, cryopreservation and maintenance have been analysed for four species groups; bivalve (Pacific, Sydney rock and pearl oysters), prawn (tiger prawn), abalone (greenlip and blacklip abalone) and finfish (salmon and barramundi), assuming the service is established at a commercial hatchery.

Key factors that were included or considered in the cryopreservation cost analysis were: equipment depreciation, labour, operation, materials, biological parameters (survival rates at different developmental stages, fertility of cryopreserved sperm at a recommended sperm:egg ratio, generation interval, number of individuals per family required at the initiation of performance evaluation, size of the breeding nucleus - number of full-sib families per generation), estimated duration for sperm collection and cryopreservation, configurations of cryopreservation equipment and cryostorage facilities. The analysis shows that cryopreservation costs per family unit (male) remain very consistent with the changes in the size of the breeding nucleus from 50 to 400 full-sib families (Figure 2) per generation for each species group considered. However, in species groups whose gametes are collected via natural spawning such as abalone the costs are about 3 times higher (about \$145/family unit) compared with species groups where strip spawning is applied (about \$55/family unit) such as prawn, bivalve and finfish.

Key factors included or considered in the maintenance cost analysis were: equipment depreciation, labour and materials (mainly liquid nitrogen). The analysis indicates that the annual maintenance costs per family unit (male) decrease with an increase in the size of the breeding nucleus from 50 to 400 full-

sib families per generation, from approximately \$260 to \$86 with an increase in family numbers from 50 to 150. The costs then decrease slowly with a further increase in the numbers of families per generation, reaching \$43 at 300 families, the largest nucleus size anticipated in the industry survey.

According to the biological parameters and facility configurations used in this study, up to 607,500, 84,375,000 and 3,375,000 fertilised eggs can, theoretically, be produced from the 0.5 mL straws stored in one dewar for finfish, bivalve and abalone groups, respectively.

2. Options to establish a cryobanking service for aquaculture selective breeding programs in Australia

A stepwise approach to establishing a cryobanking service for the aquaculture selective breeding programs and aquaculture industry has been suggested based on results from cost analyses and existing cryobanking facility investigations conducted in this study. It is anticipated that this kind of approach would not only meet the current demand in assisting desired mating in selective breeding programs but also address the technical gaps and improvements identified to achieve what has been anticipated from the proposed cryobanking service.

Alternatively, an independent facility to meet the needs of the aquaculture industry in Australia and address the technical gaps identified could be considered if funding was available (Section 9).

3. Technical gaps and/or improvements

The main technical gaps identified in establishing a cryobanking service for the aquaculture industry and improving current technologies can be summarised into the following three areas:

- A) Quality control measures to ensure the amount of sperm cryopreserved from individual males is sufficient to produce the required number of progenies in the breeding program.

- B) Development of techniques necessary to reconstruct the breeding nucleus so that genomes achieved in both sexes can be recovered if necessary.
- C) A cost benefit analyses to ensure a proposed cryobanking service is cost effective.

14. CONCLUSION

This project focused on the need to investigate cryobanking options for AS CRC partners investing in genetic improvement programs.

Specifically, the project considered options for establishing this service by evaluating published cryopreservation methods and existing cryobanking facilities in Australia and overseas, investigating costs associated with establishing an on-site cryobanking service (both cryopreservation and storage) with back up storage off-site and cost differences among potential options, and conducting a survey in the aquaculture sectors with a selective breeding program. A review of the industry's short, medium and long term requirements and a review of current technologies have identified technical gaps that need to be the focus of future R&D to improve the viability of cryopreservation for aquaculture selective breeding programs in Australia.

Model results, assessing cryopreservation and maintenance costs, assuming the proposed service was established at a commercial hatchery, demonstrated that cryopreservation costs are mainly affected by gamete collection methods, while annual costs are largely determined by the number of family units stored. Cryopreservation costs in natural spawning species such as abalone are about 3 times higher (about \$145/family unit) than costs associated with strip spawned species (about \$55/family unit). Annual maintenance costs however, vary with the number of families stored, with costs decreasing as the number of families increases.

A review of current options available for establishing a cryobanking service for aquaculture breeding programs in Australia has identified that a stepwise approach involving in a research or an existing cryobanking facility would be the most practical option available to the industry at this time. This approach would best address the concerns, considerations and technical gaps raised so far and at the same time provide immediate access to the required service.

The key technical gaps identified in providing this service can be summarised in two categories: quality control measures needed to achieve the desired outcomes with the systems defined and the techniques required to reconstruct the breeding nucleus.

Quality control measures are needed as cryopreservation protocols published to date have not been standardised and data on individual and temporal variations in gamete qualities is not available for most species investigated. Improvements in techniques to reconstruct the breeding nucleus are also critically needed as Pacific oysters is currently the only species where techniques to preserve both gametes exists, leaving other species in breeding programs vulnerable to losing the genetic advantage stored in females. Even in Pacific oysters, the performance of progenies produced from both cryopreserved gametes is yet to be investigated and needs to be established to fully secure the breeding nucleus in breeding programs.

Finally it is the projects recommendation that if a cryobanking service was to be integrated into a commercial hatchery, a cost benefit analysis would be needed to determine the correct balance between investments in a cryobanking service, broodstock management, and short- and long-term production goals.

15. REFERENCES

- ABARE, 2009. Australian Fisheries Statistics 2008
<http://www.frdc.com.au/commercial/fishstats/Australian%20Fisheries%20Statistics%202008.pdf>
- Acosta-Salmon H, Jerry DR, Southgate PC, 2007. Effect of cryoprotectant agents and freezing protocol on motility of black-lip pearl oyster (*Pinctada margaritifera* L.) spermatozoa. *Cryobiology* 54: 13-18.
- Adams SL, Hessian PA, Mladenov PV, 2003. Flow cytometric evaluation mitochondrial function and membrane integrity of marine invertebrate sperm. *Invertebrate Reproduction & Development* 44: 45-51.
- Adams SL, Smith JF, Roberts RD, Janke AR, Kaspar HF, Tervot HR, Pugh PA, Webb SC, King NG, 2009. A simple method for freezing Pacific oyster (*Crassostrea gigas*) sperm in quantities suitable for commercial hatcheries. In: Cabrita E, Robles V, Herraez P (eds), *Methods in Reproductive Aquaculture: Marine and Freshwater Species*. CRC Press, Taylor & Francis Group, USA. p. 493-496.
- Adams SL, Smith JF, Roberts RD, Janke AR, King NG, Tervit HR, Webb SC, 2008. Application of sperm cryopreservation in selective breeding of the Pacific oyster, *Crassostrea gigas* (Thunberg). *Aquaculture Research* 39: 1434-1442.
- Akarasanon K, Damrongphol P, Poolsanguan W, 2004. Long-term cryopreservation of spermatophore of the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Aquaculture Research* 35: 1415-1420.
- Alderson R, Macneil AJ, 1984. Preliminary investigations of cryopreservation of milt of Atlantic salmon (*Salmo salar*) and its application to commercial farming. *Aquaculture* 43: 351-354.
- Alfaro J, Ulate K, Vargas M, 2007. Sperm maturation and capacitation in the open thelycum shrimp *Litopenaeus* (Crustacea: Decapoda: Penaeoidea). *Aquaculture* 270: 436-442.

- Anchordoguy T, Crowe JH, Griffin FJ, Clark WH, 1988. Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis*. *Cryobiology* 25: 238-243.
- Babiak I, Glogowski J, Dobosz S, Kuzminski H, Goryczko K, 2002. Semen from rainbow trout produced using cryopreserved spermatozoa is more suitable for cryopreservation. *Journal of Fish Biology* 60: 561-570.
- Bailey JL, Lessard C, Jacques J, Breque C, Dobrinski I, Zeng W, Galantino-Homer HL, 2008. Cryopreservation of boar semen and its future importance to the industry. *Theriogenology* 70:1251-1259.
- Bart AN, Choosuk S, Thakur D, 2006. Spermatophore cryopreservation and artificial insemination of black tiger shrimp, *Penaeus monodon* (Fabricius). *Aquaculture Research*, 37: 523-528.
- Bielanski A, 2005a. Experimental microbial contamination and disinfection of dry (vapour) shipper dewars designed for short-term storage and transportation of cryopreserved germplasm and other biological specimens. *Theriogenology* 63: 1946-1957.
- Bielanski A, 2005b. Non-transmission of bacterial and viral microbes to embryos and semen stored in the vapour phase of liquid nitrogen in dry shippers. *Cryobiology* 50: 206-210.
- Bielanski A, Bergeron H, Lau PCK, Devenish J, 2003. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology* 46: 146-152.
- Blaxter JHS, 1953. Sperm storage and cross fertilization of spring and autumn spawning herring. *Nature* 172:1189-1190.
- Cabrita E, Sarasquete C, Martinez-Paramo S, Robles V, Beirao J, Perez-Cerezales S, Herraiz MP, 2010. Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology* 26: 623-635.
- Caffey RH, Tiersch TR, 2000. Cost analysis for integrating cryopreservation into an existing fish hatchery. *Journal of the World Aquaculture Society* 31: 51-58.

- Chen SL, Tian YS, 2005. Cryopreservation of flounder (*Paralichthys olivaceus*) embryos by vitrification. *Theriogenology* 63: 1207-1219.
- Clarke GN, 1999. Sperm cryopreservation: is there a significant risk of cross-contamination? *Human reproduction* 14: 2941-2943.
- Cloud JG, Miller WH, Levandusk, 1990. Cryopreservation of sperm as a means to store salmonid germ plasma and to transfer genes from wild fish to hatchery populations. *The Progressive Fish-Culturist* 52: 51-53.
- Cryogenetics. Preserving Aquatic Genes. <http://www.cryogenetics.com/>
- CSIRO. An Atlantic Salmon Selective Breeding Program for Tasmania. <http://www.csiro.au/files/files/p7hh.pdf>
- Damrongphol P, Akarasanon K, 2009. Techniques for cryopreservation of spermatophores of the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). In: Cabrita E, Robles V, Herraes P (eds), *Methods in Reproductive Aquaculture: Marine and Freshwater Species*. CRC Press, Taylor & Francis Group, USA. pp. 509-514.
- Dong QX, Huang CJ, Eudeline B, Tiersch TR, 2007. Cryoprotectant optimization for sperm of diploid Pacific oysters by use of commercial dairy sperm freezing facilities. *Aquaculture* 271: 537-545.
- Dong QX, Huang CJ, Eudeline B, Tiersch TR, 2009. Cryopreservation of Pacific oyster sperm. In: Cabrita E, Robles V, Herraes P (eds), *Methods in Reproductive Aquaculture: Marine and Freshwater Species*. CRC Press, Taylor & Francis Group, USA. pp. 487-491.
- Dreanno MS, Fauvel C, Cosson J, Billard R, 2000. Cryopreservation of sperm in marine fish. *Aquaculture Research* 31: 231-243.
- Dziewulska K, Rzemieniecki A, Czernaiwski R, Domagala J, 2011. Post-thawed motility and fertility from Atlantic salmon (*Salmo salar* L.) sperm frozen with four cryodiluents in straws or pellets. *Theriogenology* 76: 300-311.

- Fryer JL, Bartholomew JL, 1986. Established and emerging infectious diseases of fish: As fish move, infections move with them. *American Society for Microbiology News* 62: 592-594.
- Gallant PK, Richardson GF, McNiven MA, 1993. Comparison of different extenders for the cryopreservation of Atlantic salmon spermatozoa. *Theriogenology* 40: 479-486.
- Gandini G, Pizzi F, Stella A, Boettcher PJ, 2007. The costs of breed reconstruction from cryopreserved material in mammalian livestock species. *Genetics Selection Evolution* 39: 465-479.
- Gwo JC, 2000. Cryopreservation of aquatic invertebrate semen: a review. *Aquaculture Research* 31: 259-271.
- Gwo JC, Chen CW, Cheng HY, 2002. Semen cryopreservation of small abalone (*Haliotis diversicolor supertexta*). *Theriogenology* 58: 1563-1578.
- Greer DS, Harvey BJ, 2004. *Blue Genes: Sharing and Conserving the World's Aquatic Biodiversity*. Earthscan, London. pp 231.
- Herman HA, 1980. Technical development: past and future. In: *Breeding cattle by the millions: NAAB and the development and worldwide application of artificial insemination* pp83-103. University of Missouri Press, Columbia, Missouri, USA.
- Hui B, Vonau V, Moriceau J, Tetumu R, Vanaa V, Demoy-Schneider M, Suquet M, Le Moullac G, 2011. Hatchery-scale trials using cryopreserved spermatozoa of black-lip pearl oyster, *Pinctada margaritifera*. *Aquatic Living Resources* 24: 219-223.
- Jodun WA, King K, Farrell P, 2006. Methanol and egg yolk as cryoprotectants for Atlantic salmon spermatozoa. *North American Journal of Aquaculture* 69: 36-40.

- Kawamoto T, Narita T, Isowa K, Aoki H, Hayashi M, Komaru A, Ohta H, 2007. Effects of cryopreservation methods on post-thaw motility of spermatozoa from the Japanese pearl oyster, *Pinctada fucata martensii*. *Cryobiology* 54: 19-26.
- Lahnsteiner F, Mansour N, 2009. Protocols for the cryopreservation of Salmonidae semen, *Lota lota* (Gadidae) and *Esox lucius* (Esocidae). In: Cabrita E, Robles V, Herraes P (eds), *Methods in Reproductive Aquaculture: Marine and Freshwater Species*. CRC Press, Taylor & Francis Group, USA. p. 373-384.
- Lahnsteiner F, Weismann T, Patzner RA, 1997. Methanol as cryoprotectant and the suitability of 1.2 ml and 5 ml straws for cryopreservation of semen from salmonid fishes. *Aquaculture Research* 28: 471-479.
- Lang RP, Riley KL, Chandler JE, Tiersch TR, 2003. The use of dairy protocols for sperm cryopreservation of blue catfish *Ictalurus furcatus*. *Journal of the World Aquaculture Society* 34: 66-75.
- Leung LKP, 1987. Cryopreservation of spermatozoa of Barramundi, *Lates calcarifer* (Teleostei: Centropomidae). *Aquaculture* 64: 243-247.
- Li X, 2004. Abalone Aquaculture Subprogram: Development of spermatozoa cryopreservation techniques for farmed abalone. South Australian Research & Development Institute. pp 60.
- Li X, 2005. Protocol for cryopreservation of sperm from greenlip (*Haliotis laevis*) and blacklip abalone (*H. rubra*). South Australian Research and Development Institute. pp 11.
- Li X, 2008. Abalone Aquaculture Subprogram: Selective breeding of farmed abalone to enhance growth rates II. South Australian Research & Development Institute. pp 102.
- Li X, 2009. Development of Techniques for production homozygous Pacific oysters. South Australian Research & Development Institute. pp 106.
- Liao X, Ge JC, Ding SY, Chen CJ, Lu QP, Huang YH, 2008. Study on cryopreservation of the sperm of freshwater prawn *Macrobrachium nipponense*

- (Crustacea, Decapoda). Journal of Nanjing University (Natural Sciences) 44: 421-426.
- Liu B, Li X, 2008. Preliminary studies on cryopreservation of Sydney rock oyster (*Saccostrea glomerata*) embryos and larvae. Journal of Shellfish Research 27: 1125-128.
- Lueng LKP, 1991. Principles of biological cryopreservation. In: Jamieson B, (Ed.) Fish Evolution: Evidence from Spermatozoa. Cambridge Press, NY, USA. p. 231-244.
- Lyons L, Jerry DR, Southgate P, 2005. Cryopreservation of balcklip oyster (*Pinctada margaritifera* L.) spermatozoa: effects of cryoprotectants on spermatozoa motility. Journal of Shellfish Research 24: 1187-1190.
- Massip A, Leibo SP, Blesbois E, 2004. Cryobiology of gametes and the breeding of domestic animals. In: Fuller BJ, Lane N, Benson EE, (eds) Life in the Frozen State. p 347-370. CRC Press, Boca Raton, FL, USA.
- McClintock S, Groeneveld E, Hiemstra S, Blackburn H, 2007. Cryobanking in Animal Genetic Resources. <http://www.fao.org/ag/againfo/programmes/en/genetics/documents/Interlaken/sidevent/3-4/McClintock.pdf>.
- Moore R, 1979. Natural sex inversion in the giant perch (*Lates calcarifer*). Australian Journal of Marine and Freshwater Research 30: 803-13.
- Narita T, Kawamoto T, Isowa K, Aoki H, Hayashi M, Ohta H, Komaru A, 2008. Effects of cryopreservation on sperm structure in Japanese pearl oyster *Pinctada fucata martensii*. Fisheries Science 74: 1069-1074.
- Negus MT, 2008. Salmonid Sperm Cryopreservation Techniques. Special Publication 167. Minnesota Department of Natural Resources, USA. pp 20.
- Li X, 2005. Protocol for cryopreservation of sperm from greenlip (*Haliotis laevigata*) and blacklip abalone (*H. rubra*). South Australian Research and Development Institute. pp 11.
- Nimrat S, Bart AN, Keatsaksit A, Vuthiphandchai V, 2008.

- Microbial flora of spermatophores from black tiger shrimp (*Penaeus monodon*) declines over long-term cryostorage. *Aquaculture* 274: 247-253.
- Palmer PJ, Blackshaw AW, Garrett RN, 1993. Successful fertility experiments with cryopreserved spermatozoa of Barramundi, *Lates calcarifer* (Bloch), using dimethylsulfoxide and glycerol as cryoprotectants. *Reproduction, Fertility and Development* 5: 285-293.
- Paniagua-Chavez CG, Jenkins J, Segovia M, Tiersch TR, 2006. Assessment of gamete quality for the eastern oyster (*Crassostrea virginica*) by use of fluorescent dyes. *Cryobiology* 53: 128-138.
- Paniagua-Chavez CG, Tiersch TR, 2001. Laboratory studies of cryopreservation of sperm and trochophore larvae of the eastern oyster. *Cryobiology* 43: 211-223.
- Pearling Industry Advisory Committee , 2005. Review of pearl oyster hatchery policy: Phase II – Policy direction. Fisheries Occasional Publication No 27, Department of Fisheries, WA. pp 57. <http://www.fish.wa.gov.au/docs/op/op027/fop027.pdf>
- Polge C, Smith AU, Parkes AS, 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* 164: 666.
- Renard P, 1991. Cooling and freezing tolerances in embryos of the Pacific oyster, *Crassostrea gigas*: methanol and sucrose effects. *Aquaculture* 92: 43-57.
- Ryan FB, Kube PD, Parkinson SA, Li X, Nell JA, 2006. Selection of genetic strategies in Pacific oysters to maximize commercial benefit. Final Report for Fisheries Research and Development Corporation. pp 55.
- Salazar M, Lezcano M, Granja C, 2009. Protocol for cryopreservation of *Penaeus vannamei* sperm cells. In: Cabrita E, Robles V, Herraes P (eds), *Methods in Reproductive Aquaculture: Marine and Freshwater Species*. CRC Press, Taylor & Francis Group, USA. p. 505-508.
- Salinas-Flores L, Paniagua-Chavez CG, Jenkins JA, Tiersch TR, 2005. Cryopreservation of sperm of red abalone (*Haliotis rufescens*). *Journal of Shellfish Research* 24: 415-420.

- Sandilands J, Drynan K, Roberts R, 2009. Preliminary studies on the enhancement of storage time of chilled milt of Atlantic salmon, *Salmo salar* L., using an extender containing the Tex-OE heat shock-simulating factor. *Aquaculture Research* doi:10.1111/j.1365-2109.2009.02354.x.
- Stoss J, Holtz W, 1983. Cryopreservation of rainbow trout (*Salmo gairdneri*): III. Effect of proteins in the diluents, sperm from different males and interval between sperm collection and freezing. *Aquaculture* 31: 275-282.
- Stoss J, Refstie T, 1983. Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. *Aquaculture* 30: 229-236.
- Tervit HR, Adams SL, Roberts RD, McGowan LT, Pugh PA, Smith JF, Janke AR, 2005. Successful cryopreservation of Pacific oyster (*Crassostrea gigas*) oocytes. *Cryobiology* 51:142-151.
- Thacker BJ, Larsen RE, Joo HS, Leman AD, 1984. Swine diseases transmissible with artificial insemination. *Journal of the American Veterinary Medical Association* 185: 511-516.
- Tiersch TR, 2000. Introduction. In: Tiersch TR, Mazik PM (Eds.), *Cryopreservation in Aquatic Species*. World Aquaculture Society, Baton Rouge. p. xix-xxvi.
- Tiersch TR, 2008. Strategies for commercialization of cryopreserved fish semen. *Revista Brasileira de Zootecnia* 37: 15-19.
- Tsai HP, Chao NH, 1994. Cryopreservation of small abalone (*Haliotis diversicolor*) sperm – Technique and its significance. *Journal of Fisheries Society of Taiwan* 21: 347-360.
- Usuki H, Hamaguchi M, Ishioka H, 1999. Cryopreservation of Pacific oyster sperm and larvae. *Bulletin of National Research Institute of Aquaculture* S1: 3-6.
- Vuthiphandchai V, Nimrat S, Kotcharat S, Bart AN, 2007. Development of a cryopreservation protocol for long-term storage of black tiger shrimp (*Penaeus monodon*) spermatophores. *Theriogenology* 68: 1192-1199.

- Vuthiphandchai V, Pengpun B, Nimrat S, 2005. Effects of cryoprotectant toxicity and temperature sensitivity on the embryos of black tiger shrimp (*Penaeus monodon*). *Aquaculture* 246: 275-284.
- Wang H, Li X, Wang M, Clarke S, Gluis M, Zhang Z, 2011. Effects of larval cryopreservation on subsequent development of the blue mussels, *Mytilus galloprovincialis* Lamarck. *Aquaculture Research* doi:10.1111/j.1365-2109.2010.02782.x
- Xue Q, 1994. Cryopreservation of embryos of scallop, *Chlamys farreri*, in liquid nitrogen. *Marine Science* 4: 44-47.
- Yamano K, Kasahara N, Yamaha E, Yamazaki F, 1990. Cryopreservation of masu salmon sperm by the pellet method. *Bulletin of the Faculty of Fisheries, Hokkaido University* 41: 149-154.

APPENDIX 1: INTELLECTUAL PROPERTY

Not applicable.

APPENDIX 2: PROJECT STAFF

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APPENDIX 3: INDUSTRY SURVEY MATERIALS

A1. Introduction

Introduction to the survey for establishing gamete cryobanking services

Cryobanking services apply cryopreservation techniques to store live biological materials in liquid nitrogen (-196°C) and revive them at any time when needed. Theoretically the materials can be stored for hundreds of years (Lueng 1991).

Cryobanking services have been used commercially for many decades in the livestock genetic improvement programs. It has helped, in association with other biotechnologies such as artificial insemination and embryo transfer, to increase the rate of progress in selective breeding programs and more efficiently disseminate the contribution of genetically superior parents to the population with reduced risk of disease transmission.

The potential benefits of cryobanking services can be summarised as follows although they might differ slightly between different aquaculture breeding programs;

1. Preserving genetic diversity to meet current and future consumer demands

Given the typically small populations held as breeding stock for aquaculture, maintenance of genetic diversity is a critical issue for many aquaculture industries. This is because future consumer demands are difficult to predict and can change greatly over time. Furthermore, with the progress of aquaculture breeding programs it is likely that genetic diversity will decline because of the natural tendency of breeding programs to select individuals of high economic merit with high levels of accuracy and because only limited broodstock can be held in the facilities available to programs.

2. Preserving genetically improved materials to minimise the impacts of potential catastrophes and disease outbreaks

Gamete cryopreservation is a secure method for the *ex situ* preservation of genetic diversity and genetically improved materials, thus providing opportunities to reconstruct the original genetic make-up and/or re-establish the improved nucleus population. For example, when the outbreak of abalone viral ganglioneuritis occurred in Victoria in 2006, the farms involved in the selective breeding program had to be destocked, resulting in the loss of their breeding nucleus and more than 7 years' effort. Had a cryopreservation program been in place to freeze gametes from selected individuals, much of the R&D effort would have been recoverable.

3. Providing backups to the live gene banks

The wild populations and the breeding populations held in the participating organisations are the live gene banks for the genetic improvement program. However, they are not secure and can be lost as the result of deteriorating environments due to climate change and/or anthropogenic activities, as well as the other reasons mentioned in the preceding section. Cryo-banking services are ideal backups for minimising these uncertainties.

4. Facilitating the implementation of desired mating in breeding programs

Desired mating is the most efficient method to minimise the increase in inbreeding while achieving the potential optimal genetic gains. However, this kind of approach is difficult to implement for most aquaculture species because it requires paired broodstock to spawn at the same time, which is very hard to manage with existing spawning techniques and sometimes facilities. In some species such as barramundi it is impossible to get the sperm and eggs of the generation in the same spawning season because the animals mature as males first and then change to females in the subsequent spawning season. The desired mating can be implemented if the gametes can be cryopreserved and revived when needed.

5. Establishing genetic linkages among generations or breeding runs to enhance the power of genetic analyses

Due to the limited resources available to many aquaculture genetic improvement programs, the same generation stocks are normally bred in batches on different farms or at different times. This reduces the power of genetic analyses because changes in animals' performances across batches can result from both genetic and environmental changes (such as maintenance, nutrition, etc). The measurement of genetic gains is another essential part of a genetic improvement program. It will provide the way to check if the selection program works, and demonstrate whether long term effort and investment is worthwhile. Similarly, changes in animals' performance over time can also be influenced by genetic and environmental changes. Therefore, separating these two components is critical for genetic improvement programs. This can be achieved by establishing a genetic linkage(s) among generations and/or breeding runs using cryopreserved gametes.

6. Enhancing out of season breeding

Most aquaculture species naturally reproduce once a year. Therefore the populations to be used for genetic improvement programs need to be established at the similar time as for commercial production. This means that genetic improvement programs frequently compete for essential resources with commercial production activities. This competition can be avoided if the genetic improvement populations are established out of season using cryopreserved gametes. Out of season breeding can also improve the efficiency of hatchery facility usage as commercial hatchery production only last a few months each spawning season.

7. Enabling hybridizing between species or strains that spawn asynchronously

Exploitation of hybrid vigour or heterosis is a common genetic technique to improve the performance of aquaculture species. However, the species or strains involved normally spawn at different times of the season or in different seasons. Therefore, achieving the desired crosses is very challenging. This difficulty can readily be overcome if the cryopreserved gametes (such as sperm) from one species or strain are available.

8. Efficient and biosecure transference of genetic gains with less chance of disease translocation

The transportation of cryopreserved gametes is relatively simple and can be done at low cost, and reduces the chance of disease translocations as gametes are less likely to carry pathogens than whole live animals. Furthermore, if the assessment of pathogens and biosecurity risks is required the cryopreserved materials can be, prior to being transferred, examined by using sub-samples without the constraint of time period usually available. Generally it is also easier and more effective to disinfect fertilised eggs than latter life stages.

9. Potential direct economic benefits

In Australia, the annual production of farmed aquaculture species with existing or planned genetic improvement programs (e.g. barramundi) are valued at several hundred million dollars. If 1% improvement in their production efficiency can be achieved through the assistance of cryopreservation services, this would result in at least a million dollars direct benefit to industry per annum.

10. Potential benefits to wild fisheries

Benefits to wild fisheries sectors will be derived through the expansion of gamete cryobanking services into a cryogenic gene bank, which can provide a valuable tool to preserve subspecies, varieties or geographically isolated subpopulations or stocks that are in danger of extinction or contamination due to deteriorating environments and/or climate changes and anthropogenic activities.

Currently generic commercial cryobanking services are not available for any aquatic species in Australia and for most species in the world (Lang *et al* 2003; Greer & Harvey 2004; Dong *et al* 2007). The major barriers that could potentially inhibit the application of cryobanking services in aquaculture in Australia are:

- a) high initial investment and subsequent maintenance costs;

- b) the relatively small industry size of each aquaculture sector; and
- c) the availability of standardised cryopreservation protocols.

The first two barriers could theoretically be overcome if the use or establishment of a specialized cryobanking centre(s) proves to be effective and practical, and all or most aquaculture breeding programs in Australia share one or a few cryobanking centres. Most existing centres can provide access to standardized procedures and expensive equipment such as labelling systems, automated straw fillers, bulk-freezing chambers, and storage and inventory capabilities.

Some promising results have been reported using commercial dairy sperm freezing facilities to cryopreserve sperm from a couple of aquaculture species in the USA (Lang *et al* 2003, Dong *et al* 2007). However, it is not sure if the freezing settings and gamete transportation methods at these cryobanking centres will suit other aquatic species. In Norway a cryobanking centre specific for finfish sperm cryopreservation has been established to service the aquaculture industries in northern Europe. Its business is based on a model that customers collect and ship the milt to their laboratory where the cryopreservation and cryostorage are performed by the experienced staff. They have developed freezing protocols and equipment for 3 species including the Atlantic salmon (*Salmo salar*). One of their challenges is the quality control at thawing and fertilisation on commercial farms. Currently they are developing techniques for other finfish species (such as Pacific salmon).

An alternative option is to conduct on-farm cryopreservation and then send the frozen gametes to the specialised centre(s) for cryobanking. This option will require very skilled on-farm technicians and might not be practical for processing large numbers of samples. In addition, the standardised quality control measures may be difficult to implement because the specialised equipment required is not normally available at commercial on-farm hatcheries.

All these issues need to be well assessed before the best or most practical option for establishing gamete cryobanking services for priority aquatic species in Australia can be recommended, and reliable and sustainable services be developed.

This survey is designed to facilitate these assessments. It is anticipated it could provide enough information to predicate each industry's specifications for the proposed gamete cryobanking services, which require information on:

- A. The expected sample sizes (number of samples and number of replicates per samples) needing to be cryopreserved for short, medium and/or long term.
- B. The expected germplasm type(s) (sperm, eggs and/or embryo/larvae) for cryobanking.
- C. The existing cryopreservation protocol for each germplasm type and its quality control measures.
- D. The existing industry's capability to conduct cryopreservation on farm.
- E. The existing data management system for cryopreservation.
- F. The expected management strategies (who and how to determine the storage and release of the cryopreserved germplasms).

To simplify this survey most questions are self explanatory and can be answered by ticking the box provided or providing an estimated number only. Your participation and support are most appreciated.

If you have further questions please contact:

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A2. Survey form

AS-CRC survey for establishing gamete cryobanking services

please tick the light blue cell if you agree with the question, otherwise leave it open

(the √ symbol is provided in the cell M2)

please provide an estimated number in the blue cell

please provide a description or fill in the green cell

Species	
Organisation	
Name of person completing form	
Date	

A. Expected purposes for establishing cryo-banking service

1. commercial aquaculture production	
assisting hatchery production	
translocation or distribution of stock	
2. genetic improvement program	
selective breeding	
others (please specify)	
maintaining genetic variations available to industry and/or breeding program	
genetic structure of wild or original population	
clear	
not sure	
maintaining genetic variation of existing breeding program	
founding broodstocks exist	
yes	
no	
partially	
pedigree records	
yes	
no	
partially	
estimated current breeding population size per generation	
estimated number of males	
estimated number of females	
estimated medium-term (in 10 years) breeding population size per generation	
estimated number of males	
estimated number of females	
expected long-term (maximal or optimal) breeding population size	
estimated number of males	
estimated number of females	
generation interval	
number of years	
expected replacement strategy for cryopreserving improved stock	
generation by generation	

every 2nd generation	
every 3rd generation	
others (please specify)	
not sure	
assisting designed matings	
within generations	
between generations	
between generation controls	
assisting distribution of genetic gains to industry	
sperm	
eggs	
embryos/larvae	
not sure	

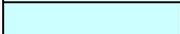
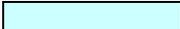
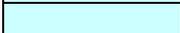
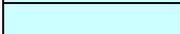
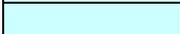
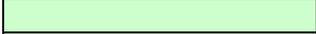
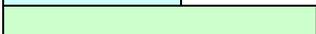
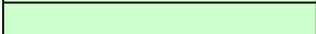
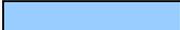
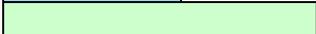
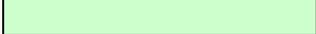
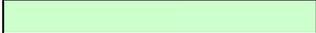
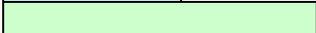
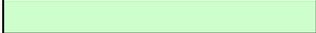
B. Germplasm cryo-banking

1. sperm

sperm collection protocol	
unpublished protocols	
protocols in public domain	
not sure	
need to sacrifice broodstock	
yes	
no	
sperm cryopreservation protocol	
unpublished protocols	
protocols in public domain	
not sure	
quality control measures	
computer assisted motility assessment	
visual motility assessment	
fertilisation assessment	
resultant progeny assessment	
staining assessment (visual)	
staining assessment (equipment such as flow cytometre)	
others (please specify)	
on site trained staff to conduct cryopreservation	
yes	
no	

2. embryos and/or larvae

cryopreservation protocols	
unpublished protocols	
protocols in public domain	
not sure	
quality control measures	
visual motility assessment	
resultant progeny assessment	
staining assessment (visual)	
others (please specify)	

on site trained staff to conduct cryopreservation	
yes	
no	
3. oocytes	
oocyte collection protocol	
unpublished protocols	
protocols in public domain	
not sure	
need to sacrifice broodstock	
yes	
no	
oocyte cryopreservation protocol	
unpublished protocols	
protocols in public domain	
not sure	
quality control measures	
visual morphological assessment	
fertilisation assessment	
resultant progeny assessment	
staining assessment (visual)	
others (please specify)	
	
on site trained staff to conduct cryopreservation	
yes	
no	
C. Cryopreservation equipment currently used by your organisation	
1. cryopreservation	
temperature controlled freezer	
others (please specify)	
	
no	
2. cryostorage facilities	
number of dewers	
others (please specify)	
	
no	
3. facilities for transportation of cryopreserved germplasms	
number of transportation dewers	
others (please specify)	
	
no	
D. Data management systems currently used by your organisation	
1. cryo-vial or straw labeling system	
manual	
automatical (please specify)	
	
2. data mangement system	

temperary	<input type="text"/>
specifically developed	<input type="text"/>
shared with breeding program	<input type="text"/>
no	<input type="text"/>
others (please specify)	<input type="text"/>
	<input type="text"/>

E. Preferred management strategy and ownership of cryostored germplasms

1. storage, release and/or replacement of germplasms are determined by

organisation depositing the germplasms	<input type="text"/>
organisation holding the germplasms (cryo-service centre)	<input type="text"/>
relevant growers association	<input type="text"/>
specific committee	<input type="text"/>
others (please specify)	<input type="text"/>
	<input type="text"/>

2. expected time period between request for storage/release and receiving of materials

time in advance for storage	
number of days	<input type="text"/>
not sure	<input type="text"/>
time in advance for release	
number of days	<input type="text"/>
not sure	<input type="text"/>

F. Existing linkage with any livestock or human cyro-banking centre(s) in Australia
if yes, please provide the following information

company name	<input type="text"/>
phone number	<input type="text"/>
street & number	<input type="text"/>
suburb	<input type="text"/>
state	<input type="text"/>
area code	<input type="text"/>