Development of Techniques for Production of Homozygous Pacific Oysters

FRDC Final Report (Project No. 2002/204)

Xiaoxu Li

March 2009

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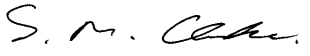

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2. NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED TO DATE

The results from this study showed:

1. The genetic load or the number of lethal equivalents in Pacific oysters was high, highly variable, and expressed progressively with larval development. On day 20 post-fertilisation (one day prior to settlement induction), the average number of lethal equivalents per individual was 11.5 or 15.0 according to the two methods used.

   This result re-emphasised the importance of avoiding inbreeding in order to maintain reliable commercial hatchery production. Inbreeding can be minimised by using a higher number of broodstock and/or broodstock with a clearly recorded pedigree history. The importance of this practice must be recognised, especially when more and more growers in Australia farm selectively bred families.

2. Doubled haploid oyster larvae were produced by inhibiting the first mitotic division of eggs activated by UV irradiated sperm, with 99% confidence according to the number of microsatellite markers analysed and their mean recombination rates published. The chromosome doubling was induced by the combined hydraulic pressure method.

3. The number of genetic markers required to differentiate doubled haploid progenies from meiotic II gynogens is determined by their recombination rates and parental heterozygous status, and can be calculated by the equations developed in the present study. Detecting contamination from meiotic gynogenetic progenies is the major challenge to production of doubled haploids in aquaculture species.

In aquaculture species doubled haploids are defined as the progenies that are produced by inhibiting the first mitotic division of the eggs fertilised by the haploid sperm if the genetic materials of the eggs' nucleus have been denatured, or the eggs activated by the genetically denatured sperm. Doubled haploid techniques originated in plant breeding and have changed the plant breeding programs, especially those to exploit both general (additive) and specific (heterotic) combing abilities and genetic studies in recent years (Tenhola-Roininen et al. 2006). Rapid development of homozygosity with doubled haploid techniques shortens the breeding time and allows a quick response to changing breeding demands. In winter crops, for example, the time needed to commercialise a cultivar can be reduced by 50% with the use of doubled haploids (Forster and Thomas 2004). Doubled haploids are especially important in the evaluation of diversity because they fix rare alleles and aid the efficient selection for quantitative traits in breeding (Tenhola-Roininen et al. 2006). For example, it enables accurate phenotyping by replicated trials at multiple sites (Forster and Thomas 2004). The additive effects among doubled haploids are twice as large as in the heterozygous diploid genotypes (Ceballos et al. 2004). In outcross species, doubled haploids enable eliminating recessive genes from lines at any breeding stage (Forster and Thomas 2004, Tenhola-Roininen et al. 2006) because a sizeable genetic load may prevent large and sustainable genetic gains (Ceballos et al. 2004). In addition, clone lines could also assist with the improvement of some traits (for instance, traits requiring destructive measurement such as most quality traits) because measurements on genetically identical sibs are equal to repeated measurements on the same individual. Selective breeding programs involved in doubled haploids are similar to their corresponding diploid counterparts except that doubled haploids are used throughout the testing and crossing procedures (Griffing 1975). In plant breeding programs exploiting both additive and non-additive genetic variances, the incorporation of doubled haploids produce, theoretically, a new wave of improved single-crosses which can be immediately evaluated and used for commercial purposes in each succeeding breeding cycle (Griffing 1975). This is not the case in its corresponding diploid counterparts.

In Pacific oysters, substantial degrees of heterosis in commercial important traits have been confirmed and the non-additive genetic component of yield variance is often the largest (Hedgecock and Davis 2007), suggesting an advanced breeding program to

exploit both additive and non-additive genetic variations could be established if doubled haploids could be created efficiently.

This FRDC project was a pilot study to evaluate two of the primary issues determining the introduction of doubled haploid technique into commercial oyster breeding. The project was scheduled for two years from July 2002 to June 2004, with the first year (first naturally spawning season) focusing on genetic loads and the second year on the method(s) for doubled haploid production.

The 1st project objective was to determine the genetic loads of Pacific oysters farmed in Australia, and this was achieved. Results from the experiments with selfed mating design revealed that:

- The genetic load of Pacific oyster is high, ranging from 11.0 to 24.5 when the relative larval survival rate was calculated relative to outcrossed family line produced by fertilising eggs pooled from 5 females with the sperm used for selfed family (pooled eggs controls); or from 7.5 to 21.1 when this rate was estimated relative to outcrossed family line established by fertilising the eggs used in selfed family with the fresh sperm pooled from 5 males of the same stock (pooled fresh sperm controls).
- The lethal equivalents (genetic loads) in Pacific oysters were expressed progressively with larval development.
- Inbreeding depression in larval shell length was highly variable between individuals, with no inbreeding depression in 2 selfed families while the other 2 showed significant inbreeding depression at day 20 post-fertilisation.
- Larval shell lengths differed significantly between different selfed families at day 20 post-fertilisation.
- In 2 of the 4 selfed families, the larval shell lengths in pooled eggs controls were significantly larger than pooled fresh sperm controls at day 20 post-fertilisation.
- The sperm from different individuals survived the extended one year liquid nitrogen storage differently, with the highest fertilisation rate being 60%.

- Self fertilisation employing cryopreserved sperm is feasible and can be used in Pacific oyster breeding programs to preserve genetic materials, develop inbred lines and exploit hybrid vigour.
- To minimise the potential negative inbreeding effects on larval growth and survival for commercial productions, oyster hatcheries should select or use the broodstock with a clear breeding history to avoid mating relatives.
- At about 1 year post-fertilisation the gonad of selfed progenies larger than 40 mm in length was well developed, and could be sexed.
- The methods used for inducing oyster metamorphosis in commercial hatcheries were not suitable for our proposed experiments, in which the maintenance of all sizes of larvae is required.

The 2nd project objective was to optimise the technologies for producing pure and/or inbred lines, and this has also been achieved. It should be clarified that pure lines can not be developed in one generation by any method, including the proposed doubled haploid one, if the parents used are heterozygous. With the doubled haploid method, it will take one generation to produce pure inbred founders, and another generation to establish pure inbred lines (which could also be achieved by other existing methods such selfing, meiotic gynogens etc). Therefore, this objective should be redefined as, "to optimise the technologies for producing pure inbred founders".

In total 49 families were produced during the 2003/04 summer period. The results indicate that:

- Four min UV irradiation was effective in denaturing the stripped spawning Pacific oyster sperm under the parameters (sperm density and UV source) used in this study.
- The first mitotic division of eggs activated by UV irradiated sperm have been successfully blocked using the combined hydraulic pressure method. Preliminary results from DNA analysis of nine, 15 day old larvae, from one of these families, revealed that 4 were homozygous on all the amplified loci (4 to 5), whereas the female broodstock used to produce these larvae were heterozygous at all these loci. This suggests that about 40% of the larvae produced in this family could be 'pure' homozygous with more than 99%

confidence, based on the average recombination rate of the markers (microsatellites and allozymes) published so far. During the 2003/2004 summer period, a total of 33 families (the first 33 families) were produced using this experimental setting.

- After the combined pressure treatment, the average rates of activated eggs surviving to 3 to 4 days and to 20 days post-fertilisation were 1.2435% (0.0182% to 6.623%) and 0.0974% (0.0094% to 0.4857%), respectively. These rates were well within the ranges predicted in the genetic load study (for details refer to chapter 7).
- Of the "doubled haploid" families produced by the above mentioned method, 19 reached the metamorphic stage (day 20 post-fertilisation), and the other 14 were discontinued due either to low survival rates or being lost by accident. The number of 20 day old larvae varied from 500 to 9,000 (4338 on average). High variation between families was expected due to high variation in the number of genetic loads per individual revealed in the genetic load experiments.
- High mortalities were also experienced during metamorphic period; the number of individuals per family decreased to an average of 7 (0 to 39). These results further confirm that the metamorphic method used for commercial production would not be suitable for managing stock with a high inbreeding level ($F \geq 0.5$). The "weak" or highly inbred oyster larvae might be very sensitive to epinephrine treatment.

Finally, the equations developed in the present study for estimating the number of genetic markers required to differentiate doubled haploid progenies from meiotic II gynogens is critical for commercial application of doubled haploid techniques and could be applied in similar assessments of other aquaculture species (with eggs being ovulated prior to the second meiotic division).

The third objective was to maintain the progenies produced. This has been achieved partially. The numbers of larvae surviving to day 20 post-fertilisation were low (about 1% on average) and highly variable (from 500 to 9,000), which were within the expectations estimated according to the number of genetic loads per oyster identified by this study. However, heavy mortalities were experienced at the metamorphic stage

in both selfed and doubled haploid families, resulting in very few spat (< than 10) in most doubled haploid families. Whereas the metamorphic rates in another experiment conducted at the same time on the effects of cryopreserved sperm in this species (Zhang 2004) were approximately 40 times higher than this study. When metamorphic problems were experienced with the genetic load study in the 2002/03 summer, substantial project efforts and care were directed to improve their survival, although these did not result in the better survival rates of doubled haploid progenies the following summer. At the same time, variation to funding was sought, but unsuccessful, to further develop the protocols used for rearing oyster larvae and small spat in the SARDI research hatchery. The spat produced by this project were sent to a commercial oyster nursery and then on to leases for grow-out.

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4. BACKGROUND

At present, most Pacific oysters are grown from wild broodstock which have been subjected to little, if any, genetic improvement. Consequently, oysters grown on leases are genetically very variable and show characteristics which do not differ substantially from the "wild-type". Selective breeding of oysters is likely to produce benefits equivalent to those already seen in horticulture and animal husbandry, resulting in significantly better quality oysters. Breeding programmes based upon traditional methods to exploit both additive and non-additive genetic improvements at the same time are slow, however, and therefore it could take many years before the industry receives benefits from this approach. In most modern plant breeding programmes the ultimate goal is to produce F1 hybrids which have superior performances and uniformities in commercially important traits. During the breeding process new further improved F1s will be developed as a result of the program. However, before F1 hybrids can be produced, at least 2 inbred lines must be created by a process of a few cycles of inbreeding, crossing, performance testing and selection if traditional methods are applied. In oysters, this process would take decades to achieve the required inbred lines.

A recently developed technique for generating doubled haploid aquaculture species offers the opportunity to produce inbred lines of oysters in only the second generation of a breeding programme (i.e. after 4 years) (Komen and Thorgaard 2007). Clearly, this has the potential to speed up the production of F1 hybrids significantly. This approach has been successfully applied in plant breeding where doubled haploid inbred lines are produced more rapidly and genetic resources utilised far more effectively than by traditional methods (Griffing 1975, Cherif et al. 2007). Doubled haploid techniques have now been applied to over 250 crop species (Forster and Thomas 2004). The proposed research will focus on the development of the methods to produce mitotic gynogenetic oysters. If it proves to be successful, the next project (or extended project) will apply this technology to establish Pacific oyster broodstock in order to produce inbred lines of oysters. These oysters will then be subjected to selective breeding protocols to produce a variety of different F1 hybrids.

The F1 hybrid oysters will have characteristics which have been selected to maximise profitability, market share, and/or customer satisfaction and also the benefit of genetic uniformity (all individuals are genetically identical).

Four shellfish selection programmes involving Pacific oysters as a major component have been initiated in the world: two joint university-industry research programs funded by the US Department of Agriculture (USDA), the Molluscan Broodstock Program and the USDA Western Regional Aquaculture Center project (Hedgecock and Davis 2007), the GENEPHYS in Europe (http://www.ifremer.fr/crema/prog_europeens/Genephys/Genephys.htm), and the Oyster Genetic Improvement Program in Australia (Ward et al. 2005). The researchers involved in the US programs have approached their breeding objectives in two ways – the selective breeding method (the Molluscan Broodstock Program) and the crossbreeding method with inbred lines (the USDA Western Regional Aquaculture Center project). A few hundred oyster families have been established and 10 to 20% gains per generation have been achieved for general yield across different rearing environments (Langdon et al. 2003). At the same time, promising results came from their crossbreeding project, and the degree of heterosis found in this species has not been observed in other farmed fish species (Hedgecock and Davis 2007). Controlled crosses among inbred lines of the Pacific oyster revealed much hybrid vigour or heterosis for larval and adult growth rates. The hybrid larvae grew faster and set 4-5 days earlier than larvae from commercial control spawns (Hedgecock et al. 1995). More importantly, the only commercial-scale trial they have conducted to date suggested that the hybrid they used was performing better than current industry stock, even though the cross was clearly inferior to several other hybrid combinations (Hedgecock and Davis 2007). These hybrid superiorities might be because they inherit dominant alleles that mask deleterious recessive mutations in many functional genes. This means that the deleterious recessive functional genes in Pacific oysters should be eliminated first in order to enhance the performance in a breeding program. The broodstock they used were made by sibling matings.

Therefore, if successful, the proposed project should be more efficient in exploiting hybrid vigour or heterosis because the doubled haploid methods to be used have been proven to be the most efficient way to create inbreeding lines for terrestrial species

(Cherif et al. 2007).

The proposed doubled haploid method has been used regularly in the plant breeding industry and has been tried in fish breeding and research over the last couple of decades. No questions in relation to GMOs have been raised by the public because the technique does not involve gene translocation.

APPLICATION HISTORY

In 1998 the project "Breeding F1 hybrid oysters from doubled haploids" was approved by the FRDC. However, the project was never initiated. After consultation with the South Australian Oyster Growers Association, which strongly supported this R&D initiative, and the FRDC, the proposed new project was cut back to two years and focused exclusively on the evaluation of methodologies to produce the doubled haploid founders.

5. NEED

A major problem facing Pacific oyster growers throughout southern Australia is maintaining consistent product quality. High level of genetic variability in oysters causes considerable variation in several economically important traits such as growth rate and condition. Variation in growth rate means extra work due to the need for frequent grading, while variation in condition within a batch may prevent growers from obtaining a market for their oysters. Even for those oysters which are of sufficient quality to reach the market, supplying oysters that vary in condition, or colour for example, creates a perception of poor quality control and undermines market confidence in the suppliers (and hence in the growers). This variability in product quality is currently unavoidable simply because of the intrinsic genetic variation in oyster stocks.

If genetically uniform oysters could be produced, then a major source of variation in product quality would have been removed from the oyster industry. All oysters grown from a batch of genetically identical spat should respond similarly in any given environment. Consequently, considerable improvements in consistency of product quality and appearance could be achieved. These genetically uniform oysters would still show some variation in for example growth rate and degree of condition due to differences in the environments they experience – for example, oysters which had been growing at the centre of a basket or lease may gain condition more slowly than those at the edge – however, because this variation would now be solely due to environmental factors, growers would be able to rapidly identify and control these factors. This would increase consistency of product quality still further.

A second major limitation to the oyster industry at present is a lack of stock which has been genetically selected to produce commercially desirable characteristics. Given the wide variation in environmental conditions under which Pacific oysters are grown in Australia (from cold, low-salinity waters off Tasmania, to warm, high salinity waters in S. Australia) considerable opportunity exists to breed oysters which have been genetically selected to perform well in a given environment.

6. OBJECTIVES

The project objectives were to:

1. To determine the genetic loads of the Pacific oysters farmed in Australia.
2. To optimise the technologies for producing pure and/or inbred lines.
3. To maintain the progenies produced.

7. GENETIC LOADS OF PACIFIC OYSTERS FARMED IN AUSTRALIA

7.1. Introduction

It is expected that the oysters produced by the doubled haploid method should be homozygous for all genes if new mutations do not occur and the DNA fragments from denatured gametes are not incorporated into their nucleus. With a homozygous genetic make-up all the deleterious and/or recessive genes inherited from the donor parent will be expressed. Thus their survivals will be strongly influenced by the genetic load (lethal equivalents) inherited. In Pacific oyster, *C. gigas*, an average of 12.7 lethal equivalents (8-14) were found by Launey & Hedgecock (2001) in the wild founders of inbred lines by analysing the segregation ratios of microsatellite DNA markers. This suggests that if doubled haploid progenies could be produced from these founders their expected average survival rates would be approximately 0.01% (Table 7.1). This also assumes that the treatment to produce doubled haploid progenies has no or limited side-effects on survivals. If broodstock carrying 8 lethal equivalents are used, the survival rate of their doubled haploid progenies would be approximately 40 times higher than the average of outcross control. In *Ostrea edulis* 15-38 fitness associated genes were revealed by Bierne et al. (1998) although overdominance effects could not be distinguished from deleterious recessive effects in their study. If a broodstock carrying such a high genetic load is used for doubled haploid induction the chance of having enough progeny for further selection is very limited. Both these studies also revealed a substantial expression of these lethal genes at the larval stage. Therefore, minimisation of inbreeding would be critical for commercial spat production and selective breeding programs, especially if intensive larval rearing systems are used, because this could further increase the chance for cross contamination from dead individuals.

Pacific oysters are not endemic to Australia; they were introduced from Japan and have been farmed for a few decades. The broodstock currently used by commercial hatcheries are from farmed stock. The investigation into the genetic load of the oyster stock farmed in Australia would not only refine our predictions of the success of the

doubled haploid procedure for producing inbred oysters, but also help us in the development of broodstock management and selective breeding strategies.

Table 7.1. Estimated survival rates of progenies due to homozygous status produced by doubled haploid method in the broodstock with different numbers of lethal equivalents (genetic load)

No of lethal equivalents	Relative survival rate (%) *	No of lethal equivalents	Relative survival rate (%) *
1	50.000000	14	0.006104
2	25.000000	15	0.003052
3	12.500000	16	0.001526
4	6.250000	17	0.000763
5	3.125000	18	0.000381
6	1.562500	19	0.000191
7	0.781250	20	0.000095
8	0.390625	21	0.000048
9	0.195313	22	0.000024
10	0.097656	23	0.000012
11	0.048828	24	0.000006
12	0.024414	25	0.000003
13	0.012207	26	0.000001

* These rates were calculated using the last equation provided in *7.2.2.1. Determination of number of lethal equivalents (genetic loads)*.

7.2. Materials and methods

7.2.1. Experimental design

7.2.1.1. Pacific oyster sperm cryopreservation

In the 2001/2002 summer (6 months prior to the official start of this project) 2.5 years old mature Pacific oysters were imported from Smoky Bay, South Australia to SARDI Aquatic Sciences Centre in Adelaide overnight in a refrigerated container. On arrival the shell surfaces were cleaned with a brush and then rinsed in fresh seawater before being put into an acclimatization tank on a flow-through system, where the oysters were held at 18°C for 2 to 6 days and fed continuously with an algal mixture of

Isochrysis sp., *Pavlova lutheri* and *Chaetoceros calcitrans*. A hole was then drilled into the left shell of each oyster and a biopsy sample taken from the gonad using an 18-gauge needle. To avoid cross-contamination between individuals a new syringe and needle were used for each oyster. The gametes were put onto a slide, diluted with seawater and sexed under the light microscope. For males their sperm motility ratios were assessed and individuals with more than 70% motility ratio were selected. After gamete collection the hole was sealed with Blu-Tack and the oyster tagged individually. These oysters were put into another tank with the same settings as the acclimatisation tank.

The sperm from each male oyster was mixed in 0.45μm filtered seawater and left for 20 to 30 min at 18-20ºC. The debris was then separated from the sperm by passing the suspension through a 20 μm screen. The density of the sperm suspension was counted under a microscope using a hemocytometer after a 1000 time dilution of the original sperm solution. The sperm concentration was then adjusted to 5×10^7/mL.

Sperm cryopreservation was conducted according to the method developed by the author. Briefly, a cryoprotectant containing 12% DMSO (v/v) and 1.2% glycine was prepared freshly each day in 0.45μm filtered seawater (salinity 37ppt) and stored in a refrigerator at 0ºC. The sperm suspensions (5×10^7/mL) were then mixed with the cryoprotectant solution at a 1:1 ratio resulting in final concentrations of 2.5×10^7/mL sperm, 6% DMSO and 0.6% glycine. The sperm cryoprotectant mixtures were mixed thoroughly by hand as soon as the sperm suspensions were combined with the cryoprotectant solutions. A Pasteur pipette was used to transfer 1.8 mL of the mixture into 2 mL cryovials with at least 8 replicates (cryovials) for each oyster. The cryovials were placed horizontally on a tray 2.5 cm above the surface of liquid nitrogen for 10 min before being immersed in liquid nitrogen (-196ºC). The cryovials were kept in the liquid nitrogen for approximately 1 year before being used in the selfing experiments. In total sperm from 107 individuals were processed.

When the shell hole was sealed with new shell, the oysters (including all tagged males and 20 tagged females) were sent back to Smoky Bay and maintained at their original lease until next summer.

7.2.1.2. Fertilisation

In December 2002 prior to natural spawning, oysters whose sperm had been individually cryopreserved in the previous summer and the 20 females tagged from the same stock were taken back from the oyster lease at Smoky Bay and maintained at the SARDI Aquatic Sciences Centre. In January 2003 the 91 live "original male" oysters were sexed using the biopsy method described previously and 16 females were found. The first 10 females were used in the first experiment where spawning was attempted by a combination of air exposure and increased water temperature. The experiment was abandoned because drip spawning was experienced. In the second experiment strip-spawning was used with the remaining 6 females.

After the top shell (right shell) was removed, the gonadal contents from each female were aspirated into a glass Pasteur pipette and then squeezed into a 750 mL container containing 0.45 µm filtered seawater. The eggs were separated from the gonadal tissues and other debris by washing them through a 75 µm screen with a 20 µm screen underneath. The eggs were then washed with 0.45 µm filtered seawater into a new 750 mL container and incubated at 24ºC for about 1 h before being used in the experiments. Very limited eggs were collected from 1 female; therefore only 5 females were used in the selfing experiments.

Pooled eggs (Pooled egg control) from 5 females tagged from the same batch at the same time as the "original males" were collected using the above-mentioned method.

Prior to fertilization the eggs were collected on a 20 µm screen and washed into a 100 ml glass beaker using as little 0.45 µm filtered seawater as possible. The concentration of the eggs was calculated by averaging counts from three 1 mL samples of 1000 time diluted egg solutions. The required egg concentrations were produced by adding 24ºC freshly filtered seawater (0.45 µm).

The cryovials were thawed by submersion in a 58-60ºC water bath and moved into a 20ºC seawater bath as soon as they were fully melted. The 8 vials from each oyster

were emptied into a small plastic container and kept for 20-30 min before being used in subsequent experiments.

Pooled fresh sperm (Pooled fresh sperm control) were also collected using strip-spawning method from 5 tagged males which had not changed their sex and processed using the method described in the section *7.2.1.1*. However, its density was diluted to 2×10^7/mL.

When cryopreserved sperm was used each replicate (tank) was produced by fertilizing 3 mL of eggs (1,225,000 eggs per replicate) with 3 mL of cryopreserved sperm. The sperm subsample was taken from the pooled container. The pooled fresh sperm controls were established by fertilizing 3 mL of eggs with 0.5 mL of pooled fresh sperm (2×10^7/mL in density). The pooled egg controls were established by fertilizing pooled eggs with 3 mL of cryopreserved sperm.

At 4 min and 8 min past-fertilization the water volume was doubled respectively with filtered seawater. The container was then filled to 150 mL at 12 min post-fertilization. After 18 min fertilization, the eggs were washed onto a 20 μm screen to remove excess sperm. Eggs from each replicate were stocked in a 20 L conical tank. These tanks are smaller modules of the oyster larval rearing tanks used in commercial hatcheries in Australia (Figure 7.1).

Three types of crosses were produced in these experiments: 1) Self families in which fresh eggs and cryopreserved sperm from the same oyster were mated (5 oysters), resulting in 5 families (A013, A085, A089, A092 and A130) with 3 replicates in each family; 2) Pooled fresh sperm controls in which eggs used in each self family in 1) were fertilised with a mix of fresh sperm from 5 males of the same batch (had not changed sex), resulting in 5 groups; and 3) Pooled eggs controls in which a mix of fresh eggs from 5 females of the same batch (had not changed their sex) was fertilised with the preserved sperm from one of the selfed oysters, resulting in 5 groups. In total 25 groups were produced.

7.2.1.3. Larval rearing

After fertilisation eggs were stored in the conical tanks (Figure 7.1) at an initial density 61.25 eggs/mL for all treatments. The seawater used for larval rearing was filtered to 10 µm with a cartridge filter. The water temperature and water salinity in the rearing tanks were kept at 24 ± 1 ºC and 36 ± 0.5 ppt, respectively. Larvae less than 48 h old were held in a static system with fine aeration and water being changed once a day. Subsequently a flow-through water supply system was used until the completion of the last epinephrine treatment for metamorphosis. To prevent the larvae escaping from the outlet a 35 µm Banjo sieve was used for larvae less than 5 day old and then a 50 µm Banjo sieve from 5 day old onwards. The larvae were fed continuously with an algal mixture of *Isochrysis* sp., *Pavlova lutheri* and *Chaetoceros calcitrans.* The concentration of the algal mixture was adjusted according to larval age, and the same concentration was used across all experimental tanks. The tanks were cleaned with fresh water every 12 h for the first 24 h and then once a day. Due to differences in fertilisation success and larval survivorship, the concentrations of fertilized eggs and larvae at different post-fertilisation dates were highly variable between treatments (Figure 7.3) and were not adjusted to the same levels.

Due to low sperm quality after thawing, less than 1% fertilisation rate was obtained with the replicates produced with the preserved sperm from the broodstock A092. These replicates were abandoned on day 10 post-fertilisation.

Figure 7.1. Over-flow system with 30 larval rearing units constructed at SARDI.

7.2.1.4. Metamorphoses

The method developed by Coon et al. (1986) to produce cultchless oyster spat was applied. Briefly, from day 21 post-fertilisation and then every other day, larvae collected with 236 µm screens from all groups were induced to metamorphose with 10^{-4} M epinephrine for approximately 10 h. Epinephrine stock solutions of 10 times higher than the concentration used at the treatment were prepared in 0.005 N HCl in freshly filtered seawater immediately prior to use. After treatment the larvae still swimming were put back to the conical larval tank of the same group. The "metamorphosed" larvae were stocked in the upweller marked for their replicate directly (Figure 7.2). The spat were fed continuously with the same algal mixture as for larvae. The metamorphosing treatments were conducted 15 times for each group and over 30 days.

Figure 7.2. Upwelling spat rearing bottles used in this study at SARDI.

Three months later the remaining spat of different groups were transferred to the South Australian Oyster Hatchery in Port Lincoln and maintained in the spat tanks there prior to being sent to Ceduna for grow-out.

7.2.1.5. Data collection

At 5 h post-fertilization the fertilization rate for each replicate was estimated by averaging the numbers of fertilized eggs in three samples of 100 oocytes under a microscope. Eggs that had developed beyond the first mitotic division were counted as fertilized eggs.

On days 5, 10, 15, and 20 post-fertilization, larvae from each tank were collected and put into a beaker filled to 500 mL for the first two samplings and to 250 or 100 mL for the last two samplings (depending on the larval density). The number of larvae in each tank was estimated by counting larvae in a 1 mL subsample from an even suspension and at the same time the shell length was measured on 30 individuals in each replicate

tank or on all individuals if less than 30 were available.

In December 2003 the fertility of selfed progenies was checked by a destructive method using 30 randomly selected individuals. The gonad in animals larger than 40 mm was well developed and could be sexed under the microscope while the gonad in animals smaller than 40 mm was poorly developed and many of them could not be properly sexed. At that time about 30% animals in the selfed families were smaller than 40 mm.

7.2.2. Statistical analyses

7.2.2.1. Determination of number of lethal equivalents (genetic loads)

Survival rate to each post-fertilisation larval age was determined as the proportion of larvae produced from fertilized eggs per tank (replicate). For example, larval survival rate on day 5 post-fertilisation was calculated by counting the number of larvae in each tank on this day and dividing this by the number of fertilised eggs in their respective tank at 5 h post-fertilisation.

The log model developed by Morton et al. (1956) represents the correlation between the survival rate of a group of individuals with a certain level of inbreeding and the genetic load of the population this group belongs to:

$$\mathbf{S} = \mathbf{e}^{-(\mathbf{A} + \boldsymbol{F}\mathbf{B})}$$

where **S** (survival rate) is the proportion of fertilised eggs developing to day 5, 10, 15 and 20 post-fertilisation, ***F*** is the inbreeding coefficient, **A** is considered the expressed load in a randomly mating population, and **B** is the hidden or concealed load that would be expressed fully only at ***F*** = 1 (pure homozygosity). ***F*** equals 0.5 when self mating system is used.

The above model was re-arranged by Sorensen (1969), Ralls et al. (1988) and Fox et al. (2008) to determine the number of lethal equivalents (**B**) per gamete.

$$\mathbf{R} = \text{relative survival rate}$$

$$= \frac{\text{survival rate of inbred population } (\boldsymbol{F})\text{: } \mathbf{e}^{-(\mathbf{A}+\boldsymbol{F}\mathbf{B})}}{\text{survival rate of outbred population: } \mathbf{e}^{-\mathbf{A}}}$$

$$= \frac{\mathbf{e}^{-(\mathbf{A}+\boldsymbol{F}\mathbf{B})}}{\mathbf{e}^{-\mathbf{A}}}$$

$$= \mathbf{e}^{-\boldsymbol{F}\mathbf{B}}$$

The number of lethal equivalents per zygote or individual is twice the number per gamete.

The following equation was used to estimate the survival rate (**Sr**) of oyster larvae due to homozygosity produced by the doubled haploid method.

$$\mathbf{Sr} = 0.5^{\mathbf{B}} * 100$$

where **B** is the same as above.

7.2.2.2. Inbreeding depression in larval survival

The inbreeding depression for survivals ($\boldsymbol{\delta}_s$) at different numbers of lethal equivalents (genetic loads) is calculated using Ralls' et al. (1988) and Keller and Waller's (2002) equation:

$$\boldsymbol{\delta}_s = (1 - \mathbf{e}^{-\boldsymbol{F}\mathbf{B}}) * 100$$

According to Ralls et al. (1988) the $\boldsymbol{\delta}_s$ value represents the percentage of mortality in the offspring with inbreeding coefficient of ***F*** that is higher than the outbred offspring.

The effective breeding number and inbreeding coefficient are calculated according to Tave (1993):

$$N_e = (4N_m N_f) /(N_m + N_f)$$

$$F = 1/(2N_e)$$

where N_e is the effective breeding number, N_f is the number of females that produce viable offspring and N_m is the number of males that produce viable offspring, and F is the same as above.

7.2.2.3. Inbreeding depression in larval length

The inbreeding depression for larval shell lengths is calculated using Fox's et al. (2008) equation:

$$\delta_L = (\mathbf{Mean}_{outbred} - \mathbf{Mean}_{selfed})/ \mathbf{Mean}_{outbred} * 100$$

An average of two outbred controls was used in this study; 1) the pooled eggs controls which were produced by fertilising, respectively, eggs pooled from 5 females of the same stock with the cryopreserved sperm used for each selfed family, and 2) the pooled sperm controls which were produced by fertilising, respectively, eggs used for each selfed family with fresh sperm pooled from 5 males of the same stock.

7.2.2.4. General analyses

Data were analysed using SPSS 13.0 software. One-way ANOVA were applied to compare means between different treatments in the fertilisation (cryopreserved sperm vs controls) and larval length (selfed family vs controls, and between controls) assessments because the data did not show any significant departure from normality and homogeneity of variances ($P < 0.05$). However, data on larval survival rates and rearing densities did not meet these conditions so the non-parametric Mann-Whitney Test was used instead. When a significant difference was detected by ANOVA, Tukey's b multiple comparisons were used to compare means. All tests were

performed at the 5% significance level. The results are presented as means ± standard errors.

7.3. Results and discussion

Heavy mortalities were experienced during the metamorphosis stage in all groups. Therefore, only the data collected during the larval stages were analysed. It was thought at this stage that the main reason was due to the contamination from returning the unhealthy larvae back into the larval rearing tanks after metamorphosis treatment or the difficulty in maintaining proper water flow to suspend spat collected over 30 days in the same upweller.

7.3.1. Inbreeding depression (survivorship)

7.3.1.1. Fertilisation

Of the 91 live oysters brought back from the grow-out lease 16 changed their sex from male when they were used for sperm cryopreservation in the previous year to female, which is equivalent to a male to female change ratio of 17.6% after one year on growing in Smoky Bay (from 2.5 year old to 3.5 year old). However, further investigation would be required to confirm if similar level of sex change occurs in other age (size) groups, in other locations and sex change from female to male (all 5 original females opened had not changed their sex). The author's previous experiences suggest that the sex is skewed to males when Pacific oysters are mature at the first spawning season and more females appeared in old stocks although these are variable between years and localities.

The sperm used in this study had been cryopreserved for approximately 1 year in liquid nitrogen and successfully fertilised eggs produced from both same individual (sex changed in the second spawning season) and females from the same stock that had maintained their sex during this period (Table 7.2). When eggs were fertilised by the pooled fresh sperm, approximately 90% fertilisation rates were achieved in all the

selfing broodstock (Table 7.2), which was slightly lower than the fertilisation rates reported by Li (2007) and Adams et al. (2008) using the freshly strip-spawned male and female gametes for the same species, but higher than Lannan's (1971) if the same type of cross (eggs from sex changed individuals crossed by the fresh sperm from different animals) is compared. This might be due to the use of different assessment standards in these studies. For example, in this study eggs developing beyond the first mitotic division were considered fertilised whereas in Li's (2007) study the fertilisation were assessed at an earlier stage and eggs with polar body(ies) were treated as fertilised. The results from this study showed that fertilisation rates were strongly affected by the cryopreservation processing as the rates between crosses using cryopreserved sperm and the fresh sperm differed significantly ($P < 0.05$), whereas the rates between the selfed family and the outcrossed control produced by the cryopreserved sperm from the same individual were similar ($P > 0.05$) for all cases. These results also suggest that the fertilisation compatibility of the male and female gametes from the same individual after sex change in the sequential spawning season could be as good as outcrosses (Table 7.2).

Table 7.2. Fertilisation rates (%) of the selfed families and pooled eggs and pooled fresh sperm controls

Individual ID	Fertilisation rate [3]		
	Selfing [1]	Pooled eggs [2]	Pooled fresh sperm [2]
A013	5.2 ± 0.7 [a,A]	7.3 ± 0.7 [a,A]	90.3 ± 1.5 [a,B]
A085	29.2 ± 0.9 [c,A]	29.0 ± 1.2 [c,A]	89.0 ± 1.7 [a,B]
A089	21.7 ± 0.7 [b,A]	23.7 ± 1.7 [b,A]	94.0 ± 1.5 [a,B]
A130	55.0 ± 2.8 [d,A]	60.0 ± 1.5 [d,A]	91.3 ± 2.2 [a,B]

[1] The percentage was calculated by averaging the percentages across the replicate tanks (mean ± SE).

[2] The percentage was calculated by averaging the repeated counts from one tank (mean ± SE). Pooled eggs controls were produced by fertilising, respectively, eggs pooled from another 5 females of the same stock with the cryopreserved sperm used for each selfed family; and Pooled fresh sperm controls were produced by fertilising, respectively, eggs used for each selfed family with fresh sperm pooled from 5 males of the same stock.

[3] Data in the same column with different small letters differ significantly ($P < 0.001$ for all cases). Data in the same row with different capital letters differ significantly ($P < 0.001$ for all cases).

The fertilisation rates with sperm cryopreserved for one year (Table 7.2) were much lower than the rates (more than 90%) using sperm cryopreserved for 4 h (Adams et al., 2008) or less than 2 days (Li, unpublished data) for the same species. The cryopreserved sperm from individual A089 was one of 5 random samples (individuals) checked within 24 h at the freezing stage; all resulted in more than 90% fertilisation rate. Deep-freezing causes damages to sperm including the acrosome and mitochondrial membrane (details refer to Appendix 3). The result from this study also suggests that the quality of cryopreserved oyster sperm might deteriorate with the extension of the storage time in liquid nitrogen, although further confirmation would be required. In addition, after one year storage in liquid nitrogen the fertilisation rates varied significantly between sperm collected from different individuals (Table 7.2). These variations would indicate that sperm from different individuals tolerance cyro-damage differently. Wide variation in fertility between pair crosses was reported by

Adams et al. (2008) using cryopreserved Pacific oyster sperm, although pair crossing could be confounded by gamete incompatibility between some crosses if it exists in Pacific oysters.

The Pacific oyster is a dioecious bivalve, with simultaneous hermaphrodites occurring rarely, and alternating hermaphrodites more typical (Lannan 1971). In this study more than 85% of the oysters had survived the biopsy method used for sperm collection and the sperm preserved could maintain reasonable fertilisation capability for at least one year. This provides a potential technique to establish pure lines in Pacific oysters at an inbreeding coefficient accumulation rate of 50% per generation, which is the second most efficient technique (next to the doubled haploid) in pure line development.

7.3.1.2. Larval survivorship

In this study the stocking densities at early development stages varied significantly between treatments (Figure 7.3), from less than 10 to approximately 50 individuals/mL, and then reduced to less than 10 on day 20 post fertilisation in all replicates except Pooled fresh sperm controls. The highest density used in this study was much lower than the density (100 individuals/mL for 15 day old or older larvae) used in commercial production with the similar system (A Butterworth, pers. comm.). Therefore, the densities used in this study were not expected to have significant impacts on larval performances. If a negative density impact existed, the inbreeding depression would be underestimated because from day 5 post-fertilisation onward, the stocking densities in controls were higher than selfed families.

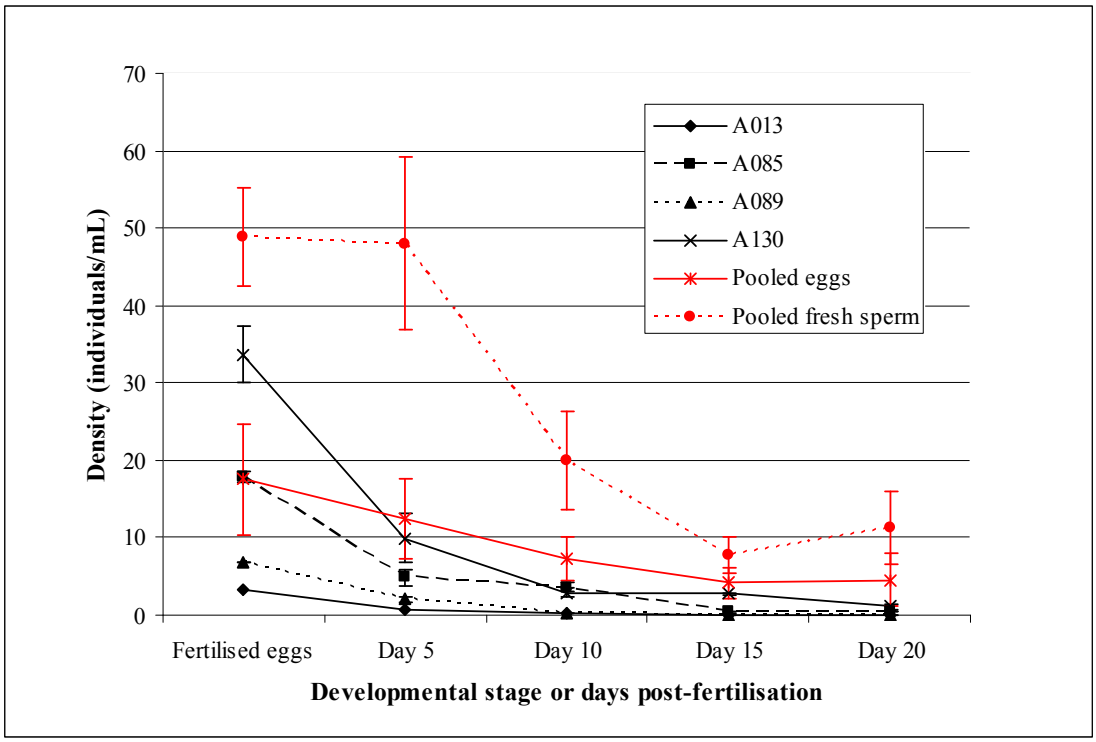

Figure 7.3. Stocking densities of fertilised eggs or larvae of different ages; A013, A085, A089 and A130 were the selfed Pacific oyster families (inbred families), the pooled eggs controls were produced by fertilising, respectively, eggs pooled from 5 females of the same stock with the cryopreserved sperm used for each selfed family; and the pooled fresh sperm controls were produced by fertilising, respectively, eggs used for each selfed family with fresh sperm pooled from 5 males of the same stock. Bars are standard error.

Differences in larval survival rates between selfed families and between selfed families and controls became larger when larvae became older (Table 7.5 and Figure 7.4). For example, the difference between families A013 and A089 increased from less than 0.5 fold on day 5 to 20 fold on day 20. Differences between selfed families and controls increased from 1 fold to at least 5 fold on day 20. These results suggest that different selfed families responded to inbreeding depression differently. However, this trend needs to be treated cautiously because the non-parametric Mann-Whitney Test did not demonstrate a significant difference within each sample date.

Table 7.3. Percentage survival rates of selfed (inbred) and outbred oyster larvae at different ages (mean ± SE) [1]

Family ID	Days post-fertilisation [4]			
	5	10	15	20
A013	20.95 ± 5.59 [a]	12.68 ± 4.91 [a]	2.20 ± 1.66 [a]	2.21 ± 1.14 [a]
A085	27.32 ± 6.16 [a]	20.14 ± 3.30 [a]	2.07 ± 0.37 [a]	2.67 ± 0.52 [a]
A089	29.89 ± 4.18 [a]	2.64 ± 0.66 [a]	0.59 ± 0.07 [a]	0.11 ± 0.04 [a]
A130	28.92 ± 6.56 [a]	8.36 ± 0.82 [a]	8.22 ± 0.74 [a]	3.24 ± 0.63 [a]
Pooled eggs [2]	66.21 ± 8.52 [a]	40.96 ± 14.11 [a]	32.49 ± 14.42 [a]	50.60 ± 23.65 [a]
Pooled fresh sperm [3]	94.99 ± 13.84 [a]	40.49 ± 10.19 [a]	13.99 ± 4.08 [a]	19.75 ± 10.73 [a]

[1] The percentage was calculated by dividing the number of live larvae at respective post-fertilisation day with the number of fertilised eggs.
[2] Eggs pooled from 5 females of the same stock were crossed with the cryopreserved sperm used to establish each of the selfed familes, respectively.
[3] Fresh sperm pooled from 5 males of the same stock were crossed to the eggs used to establish each of the selfed families, respectively.
[4] Data in the same column with different letters differ significantly ($P < 0.05$).

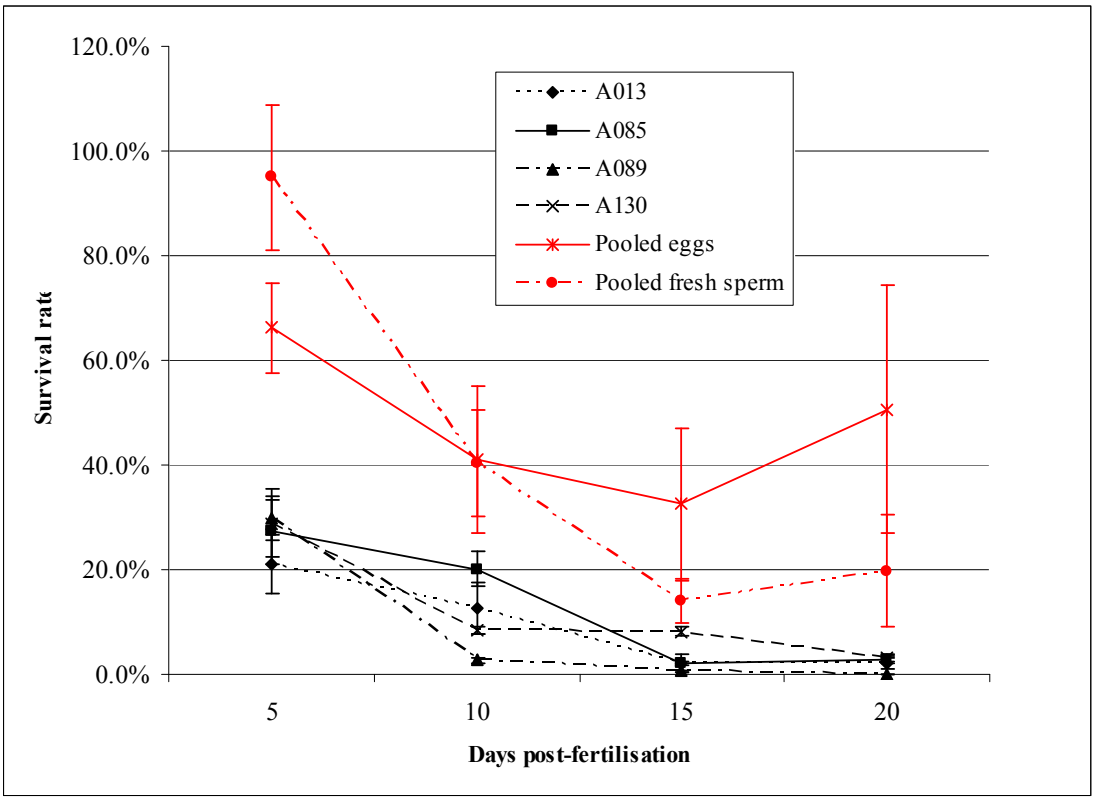

Figure 7.4. Larval survival rates at different ages: A013, A085, A089 and A130 were selfed Pacific oyster families (inbred families); the pooled eggs controls were produced by fertilising, respectively, eggs pooled from 5 females of the same stock with the cryopreserved sperm used for each of the selfed families; and the pooled sperm controls were produced by fertilising, respectively, eggs used for each of the selfed families with fresh sperm pooled from 5 males of the same stock.

7.3.1.3. Number of lethal equivalents per zygote (individual)

The number of lethal equivalents per zygote (individual) at different larval ages is presented in Table 7.4 and Figure 7.5. They were estimated relative to the survival rates of two controls (the pooled eggs control and the pooled fresh sperm control), respectively. The numbers of genetic loads calculated using different controls show a similar trend; the number of genetic loads increases with the development of oyster larvae (Table 7.4 and Figure 7.5). The average number of lethal equivalents at day 20 post-fertilisation was 15.0 ± 3.2 (11.0-24.5) when pooled eggs controls were used and 11.5 ± 3.2 (7.5-21.1) while pooled fresh sperm controls were used. The average of these two estimates is 13.3, equal to 0.66 lethal equivalents per chromosome given

Pacific oysters having 20 diploid chromosomes (2N) (Ahmed and Sparks 1967; Li and Havenhand 1998). This level of genetic load agrees with the finding by Launey and Hedgecock (2001) in the same species, with an average of 12.7 lethal equivalents (8-14) by analysing the segregation ratios of microsatellite DNA markers. Significantly high genetic loads (15-38 fitness associated genes) were revealed by Bierne et al. (1998) in *Ostrea edulis*. The studies by Bierne et al. (1998) and Launey and Hedgecock (2001) extended to the early spat stages. However, the results from this and their studies are consistent, because most developmental processes in oysters take place prior to metamorphosis (Li 1996). The study by Cohen (1992, cited from Husband and Schemske 1996) in the ascidian *Corella* showed that inbreeding depression in a selfing and an outcrossing species were similar during a late life stage (juvenile survival), but the outcrosser had significantly more inbreeding depression during an early stage (embryo survival). This suggests that purging by inbreeding might not occur uniformly throughout the life cycle. In plants, selfers exhibit most of their inbreeding depression late in the life cycle, whereas outcrossers exhibit substantial inbreeding depression at both early and late stages. If this is also applicable to oysters, it is expected that fecundities in some individuals in this species would be affected. Unfortunately, proper assessment of fecundities in selfed families was impossible, as heavy mortality was experienced during the metamorphic stage, and in the subsequent summer about 30% of the progenies in the selfed families were smaller than 40 mm and could not be sexed.

The numbers of lethal equivalents found so far in the two oyster species are larger than those reported for most other animals, and comparable to conifers; being 2.8 for mammals (mean average per individual), 4.3 for birds, 2.8 for *Drosophila*, and 8.1 for conifers (Lynch and Walsh (1998). A large genetic load is expected in highly fecund organisms (including most bivalve species), and could be attributed to the greater number of cell division to produce tens of millions of eggs or billions of sperm at each spawning season, thus increasing the chance for mutations (Williams 1975, Launey and Hedgecock 2001).

The lethal equivalents revealed by the method used in this study could be underestimated because homozygosity for two or more deleterious genes with possibly synergistic effects is more likely at high levels of inbreeding, such as the self

mating used in this study, where the coefficient of inbreeding (*F*) was 0.5 per generation.

Table 7.4. Number of lethal equivalents expressed at different ages in selfed oyster larvae

Individul ID	Days post-fertilisation [1]			
	5	10	15	20
A013				
pooled eggs[1]	4.6	4.7	10.8	12.5 [1]
pooled fresh sperm[2]	*6.0*	*4.6*	*7.4*	*9.1*
A085				
pooled eggs	3.5	2.8	11.0	11.8
pooled fresh sperm	*5.0*	*2.8*	*7.6*	*8.3*
A089				
pooled eggs	3.2	11.0	16.1	24.5
pooled fresh sperm	*4.6*	*10.9*	*12.7*	*21.1*
A130				
pooled eggs	3.3	6.4	5.5	11.0
pooled fresh sperm	*4.8*	*6.3*	*2.1*	*7.5*
Average				
pooled eggs	3.7 ± 0.3	6.2 ± 1.7	10.8 ± 2.2	15.0 ± 3.2
pooled fresh sperm	*5.1 ± 0.3*	*6.2 ± 1.7*	*7.5 ± 2.2*	*11.5 ± 3.2*
(mean ± SE)				

[1] Eggs pooled from 5 females of the same stock were crossed with the cryopreserved sperm used to establish this selfed family.

[2] Fresh sperm pooled from 5 males of the same stock were crossed to the eggs used to establish the selfed family.

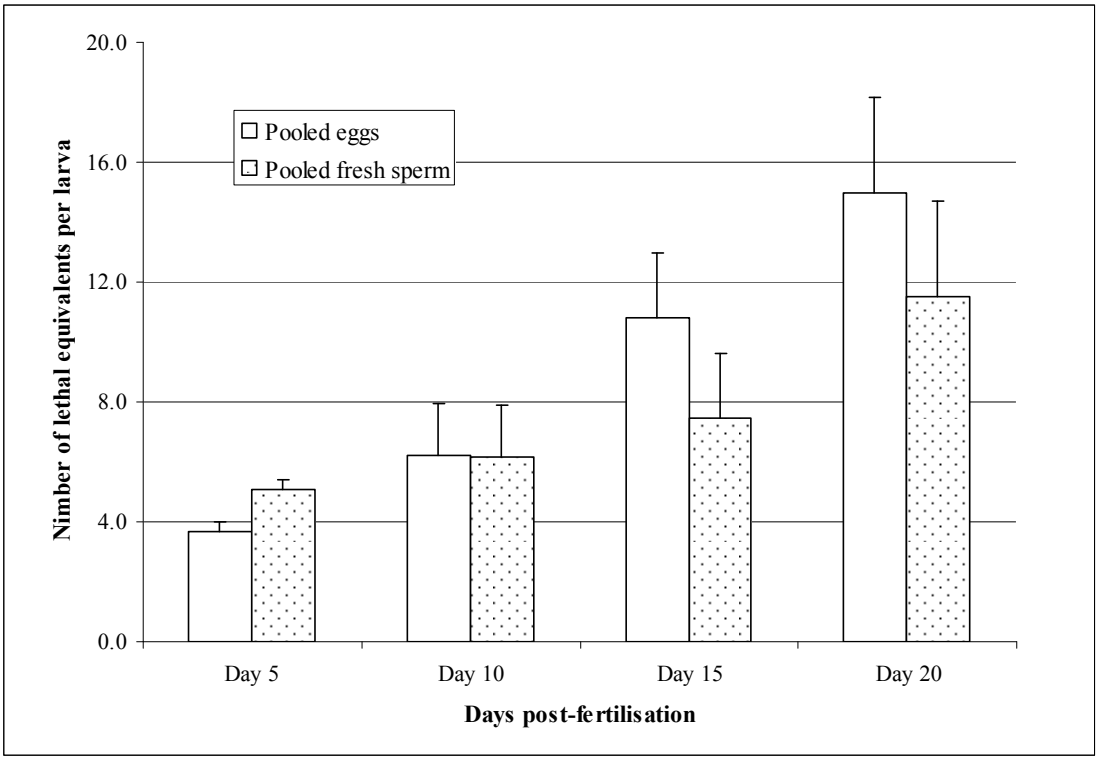

Figure 7.5. Number of lethal equivalents expressed at different ages in selfed oyster larvae.

Three oysters produced genetic loads of approximately 12 and one produced a load of 24.5 according to the estimations relative to the pooled eggs controls (Table 7.4). These results can be used to refine the predictions of the success of the doubled haploid procedure for producing inbred oysters. As a rough guideline, a genetic load of 12 lethal equivalents or lower should give an adequate survival of oyster larvae; for each ten million fertilised eggs induced to form doubled haploids, about 2442 should survive to day 20 post-fertilisation at 25^{o}C (Table 7.1). This should be enough to stand a good chance of finding healthy and fertile offspring, if more than 15% could metamorphose into spat (Zhang 2004). However, due to high variation in genetic loads reported so far for this species, as many oysters as possible would need to be put through the doubled haploid procedure, and families with low survivals be discarded, in order that the limited resources are focused on the families with good survival.

7.3.2. Inbreeding depression (larval shell length)

7.3.2.1. Larval shell length at different ages

The larval lengths of progenies produced by different crosses are showed in Figures 7.6, 7.7, 7.8, 7.9 and 7.10, and Tables 7.5. Analyses indicate that shell lengths of the larvae produced in the pooled eggs controls did not differ significantly at each sample day. They were either similar to (Figures 7.8 and 7.9 and Table 7.5) or significantly larger than (Figures 7.5 and 7.6 and Table 7.5) the other controls - the pooled fresh sperm controls. The difference in larval lengths between different crosses in some selfed families could not be explained purely by egg qualities because selfed larvae in the family A013 were significantly larger than their pooled fresh sperm control (Figure 7.6 and Table 7.5).

The results also revealed that at day 20 post-fertilisation, the larvae derived from selfing were significantly smaller ($P < 0.01$ for all cases) than both controls (the pooled eggs and the pooled fresh sperm) in the broodstock groups A085 and A130 (Figures 7.7 and 7.9). In broodstock group A013, on the other hand, the larvae resulting from selfing were significantly larger than the pooled fresh sperm control ($P < 0.01$), but did not significantly differ from the pooled eggs control (Figure 7.6). In broodstock group A089, the larval sizes in different crosses were similar ($P > 0.05$).

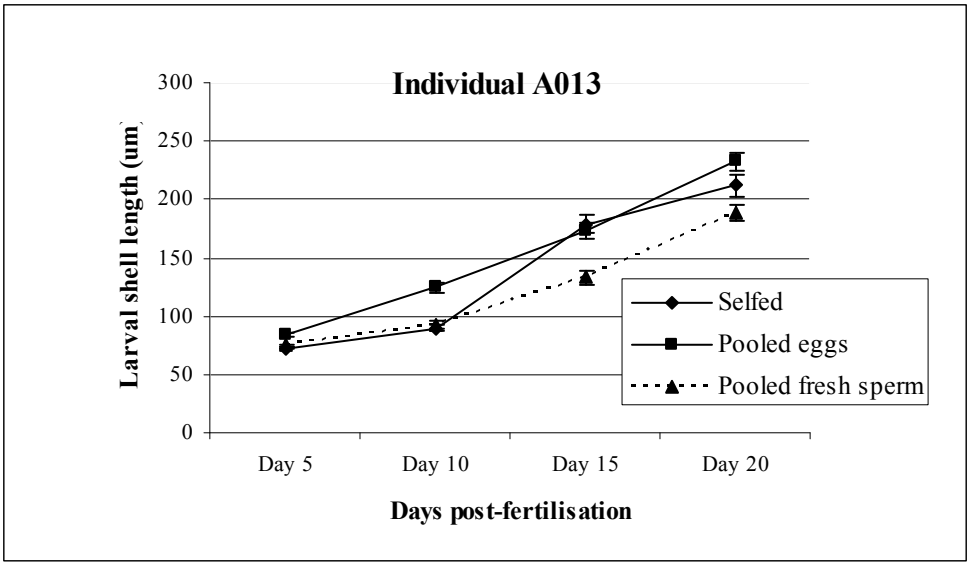

Figure 7.6. Differences in larval lengths at different ages (days post-fertilisation) of progenies produced with the broodstock A013 by different crosses, including selfing and two different outcrossings (male and female gametes from A013 were respectively fertilised with the pooled fresh gametes of the opposite sex from the same stock).

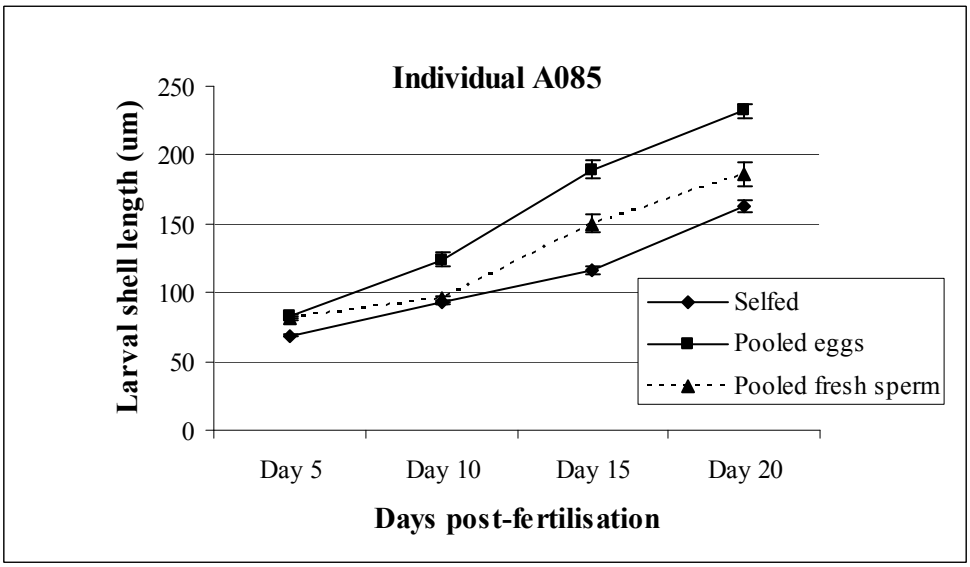

Figure 7.7. Differences in larval lengths at different ages (days post-fertilisation) of progenies produced with the broodstock A085 by different crosses, including selfing and two different outcrossings (male and female gametes from A085 were respectively fertilised with the pooled fresh gametes of the opposite sex from the same stock).

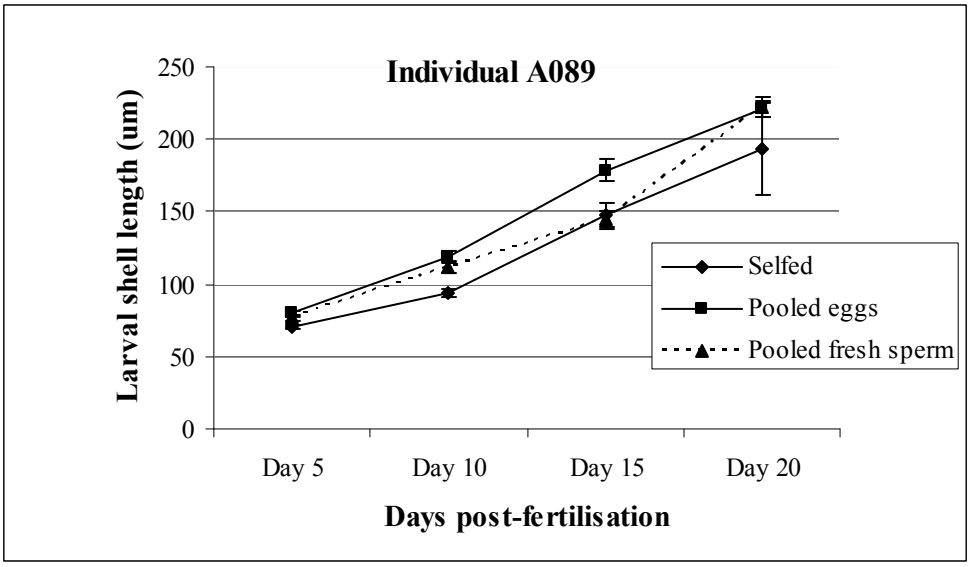

Figure 7.8. Differences in larval lengths at different ages (days post-fertilisation) of progenies produced with the broodstock A089 by different crosses, including selfing and two different outcrossings (male and female gametes from A089 were respectively fertilised with the pooled fresh gametes of the opposite sex from the same stock).

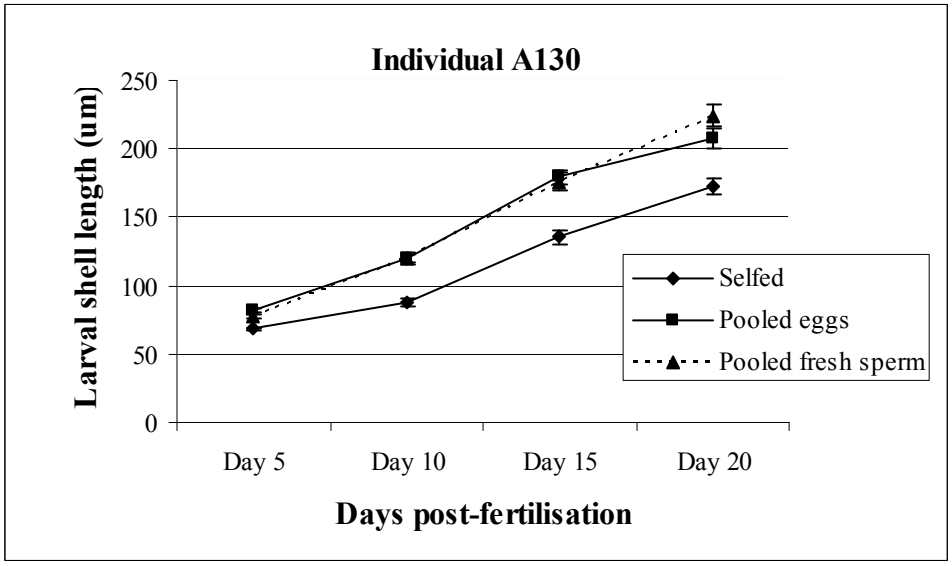

Figure 7.9. Differences in larval lengths at different ages (days post-fertilisation) of progenies produced with the broodstock A130 by different crosses, including selfing and two different outcrossings (male and female gametes from A130 were respectively fertilised with the pooled fresh gametes of the opposite sex from the same stock).

Table 7.5. Shell lengths of selfed and outbred oyster larvae at different sampling days post-fertilisation (mean ± SE)

Selfing broodstock ID	Days post-fertilisation[1]			
	5	10	15	20
A013				
Selfed	72.7 ± 1.0 [a]	89.7 ± 2.4 [a]	178.5 ± 7.9 [a]	211.9 ± 9.7 [a]
Pooled eggs [2]	83.7 ± 1.6 [b]	124.3 ± 4.0 [b]	173.0 ± 6.8 [a]	232.3 ± 8.0 [a]
Pooled fresh sperm [3]	75.0 ± 1.2 [a]	92.0 ± 3.2 [a]	133.3 ± 6.1 [b]	189.0 ± 7.0 [b]
A085				
Selfed	68.4 ± 0.8 [a]	93.1 ± 1.9 [a]	116.9 ± 2.9 [a]	163.1 ± 4.6 [a]
Pooled eggs [2]	83.3 ± 1.6 [b]	124.0 ± 4.9 [b]	189.7 ± 5.9 [b]	232.0 ± 5.4 [b]
Pooled fresh sperm [3]	82.0 ± 1.4 [b]	95.3 ± 2.6 [a]	150.7 ± 6.5 [c]	186.0 ± 8.3 [c]
A089				
Selfed	70.3 ± 0.9 [a]	93.5 ± 2.6 [a]	147.5 ± 9.1 [a]	193.3 ± 31.8 [a]
Pooled eggs [2]	79.7 ± 1.7 [b]	118.7 ± 3.6 [b]	178.7 ± 7.1 [b]	220.7 ± 5.4 [a]
Pooled fresh sperm [3]	76.7 ± 1.5 [b]	112.0 ± 4.4 [b]	145.0 ± 5.7 [a]	222.3 ± 6.9 [a]
A130				
Selfed	68.3 ± 0.8 [a]	87.5 ± 2.6 [a]	135.5 ± 5.4 [a]	172.3 ± 6.3 [a]
Pooled eggs [2]	81.7 ± 0.8 [c]	120.3 ± 4.1 [b]	179.3 ± 4.9 [b]	207.3 ± 7.7 [b]
Pooled fresh sperm [3]	78.0 ± 0.8 [b]	120.0 ± 3.0 [b]	176.0 ± 6.5 [b]	224.0 ± 7.8 [b]

[1] Within each broodstock group the data in the same column with different small letters differ significantly ($P < 0.001$).
[2] Eggs pooled from 5 females of the same stock were crossed with the cryopreserved sperm used to establish each of the selfed families, respectively.
[3] Fresh sperm pooled from 5 males of the same stock were crossed to the eggs used to establish each of the selfed families, respectively.

When larvae of different selfed families were compared, their shell lengths were similar ($P > 0.05$) on days 5 (except for A013) and 10 post-fertilisation, but differed significantly ($P < 0.05$) from the pooled eggs controls. While at each of the two subsequent sampling times (days 15 and 20), the larval sizes differed significantly between most selfed families (Figure 7.10 and Table 7.6).

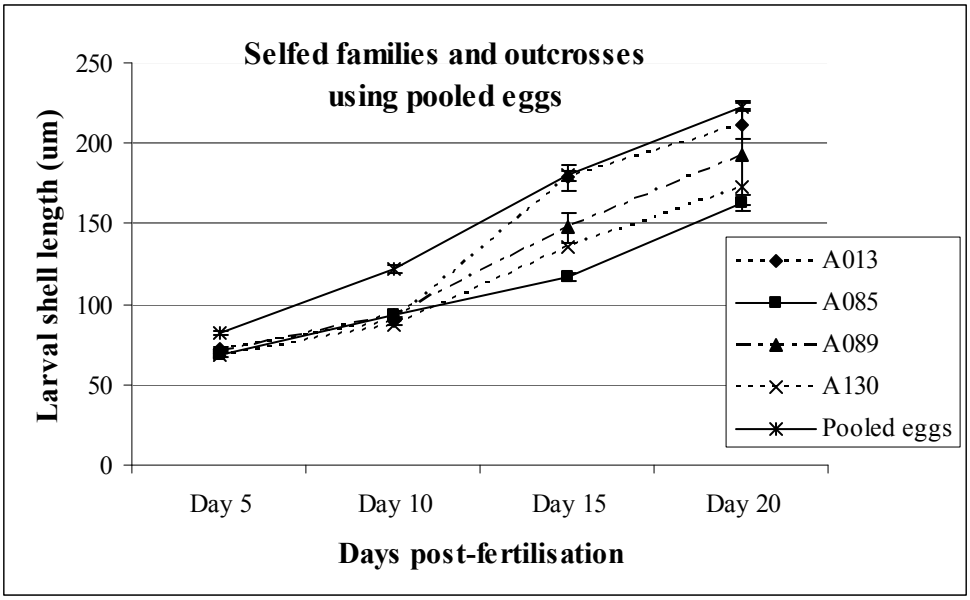

Figure 7.10. Larval lengths at different days post-fertilisation; A013, A085, A089 and A130 were the selfed Pacific oyster families (inbred families), the pooled eggs were produced by fertilising, respectively, eggs pooled from 5 females of the same stock with the cryopreserved sperm used for each of the selfed families.

Table 7.6. Shell lengths of selfed and outbred oyster larvae at different sampling days post-fertilisation (mean ± SE)

Selfing broodstock ID	Days post-fertilisation[1]			
	5	10	15	20
A013	72.7 ± 1.0 [b]	89.7 ± 2.4 [a]	178.5 ± 7.9 [c]	211.9 ± 9.7 [b,c]
A085	68.4 ± 0.8 [a]	93.1 ± 1.9 [a]	116.9 ± 2.9 [a]	163.1 ± 4.6 [a]
A089	70.3 ± 0.9 [a,b]	93.5 ± 2.6 [a]	147.5 ± 9.1 [b]	193.3 ± 31.8 [a,b,c]
A130	68.3 ± 0.8 [a]	87.5 ± 2.6 [a]	135.5 ± 5.4 [a,b]	172.3 ± 6.3 [a,b]
Pooled eggs [2]	82.1 ± 0.8 [c]	121.8 ± 2.1 [b]	180.2 ± 3.1 [c]	223.1 ± 3.5 [c]

[1] Data in the same column with different letters differ significantly ($P < 0.05$).
[2] Eggs pooled from 5 females of the same stock were crossed with the cryopreserved sperm used to establish each of the selfed families, respectively.

7.3.2.2. Inbreeding depression

The evidence presented in this study suggests that inbreeding depressions in larval shell lengths differ significantly in different broodstock (Figure 7.10). Broodstock A013 did not show any depression at later larval development stages, while various levels of inbreeding depressions were expressed throughout the larval development stages in other individuals (Figure 7.11).

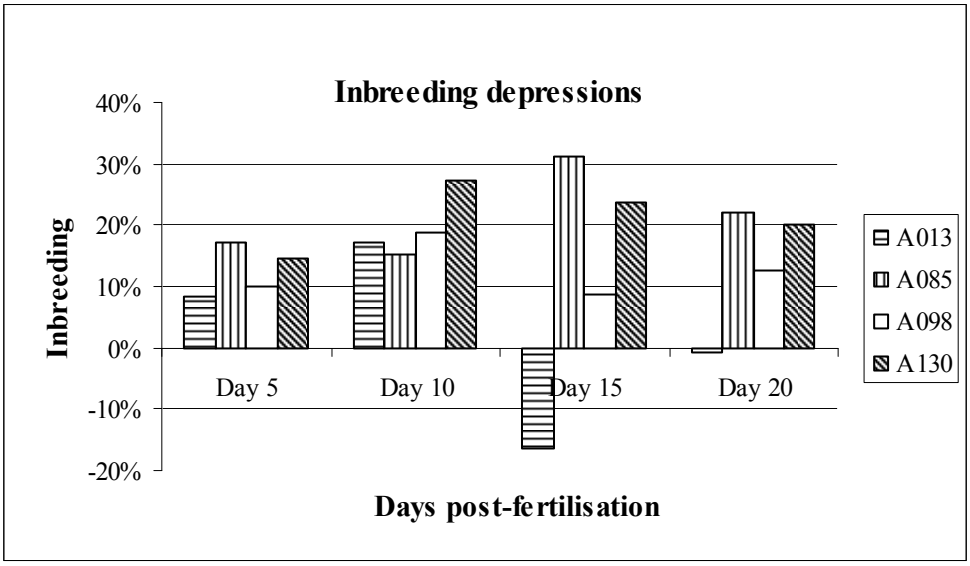

Figure 7.11. Inbreeding depression of larval lengths in the selfed families relative to the average of their outcrosses, respectively.

Self fertilisation has a coefficient of inbreeding (*F*) of 0.5 per generation, and is normally expected to produce inbreeding depression. Significant depression in growth of selfed larvae was also reported in the functional hermaphrodite, *Pecten maximus* (Beaumont and Budd 1983) and the occasional rare hermaphrodite, *Mytilus galloprovincialis* (Beaumont and Abdul-Martin 1994). Significant depression in early survival of selfed larvae was observed in *M. galloprovincialis* but not in *P. maximus*. However, no evidence of inbreeding depressions in both growth and survival during the larval stage were found in another functional hermaphrodite *Argopecten irradians* (Wilbur and Gaffney 1991).

Inbreeding effects reported in oyster species have been inconsistent. In the American oyster *Creassotrea virginica*, the larvae derived from full-sib matings (F = 0.25) showed a trend towards better larval survival in comparison to outbred larvae, and no significant depression in growth (Mallet and Haley 1983). Longwell and Styles (1973), on the other hand, found that outbred larvae were consistently larger than full sib mated inbred larvae. In Pacific oyster *C. gigas,* Hedgecock et al. (1995) compared the performance of inbred and outbred progeny obtained from matings between the offspring of selfed hermaphroditic Pacific oysters. Hybrid vigor was revealed in both larval survival and larval shell lengths. However, the results of Lannan's study (1980) showed that inbred larvae had a lower mortality than outbred larvae. In Lannan's study, inbred female parents were used, which could potentially bias the comparison because the survival of the outbred larvae may have been more adversely affected by having inbred dams than the survival of the inbred larvae (Sheridan 1997). Inbreeding history has been considered a potential reason for the low levels of inbreeding depression in a few outcrossing species (Husband and Schemske 1996).

7.3.3. Implications of the extent of genetic load found in Pacific oysters to commercial hatchery production

The effects of the accelerated rate of inbreeding in small populations used for commercial production could potentially result in substantial economical consequences to oyster hatcheries. Table 7.7 shows that if the genetic load of the broodstock and the inbreeding of resultant progenies are both high, the consequence from expected larval mortality can be serious, especially in those hatcheries where intense larval rearing systems are used. This is due to increased rearing intensity further increasing the chance of cross contamination from dead individuals. In Australia, hatcheries use broodstock selected from stocks farmed by the industry and/or from the specific designed breeding program run by themselves or Australian Seafood Industries Pty Ltd. As practiced elsewhere in the world, hundreds of broodstock were used each year to propagate farming stocks of the order of millions of individuals. In contrast to the large number of broodstock used, the effective population sizes calculated by Hedgecock and Sly (1990) on two stocks produced in US were only 40.6 and 8.9, respectively, equivalent to an inbreeding coefficient of

1.22% and 5.56%. They related these discrepancies between the number of broodstock used and the effective population sizes to: 1) insufficient numbers of broodstock used in the spawns that produced the derived stocks, 2) unequal spawning success among the individuals chosen as broodstock, 3) random, differential survival of families within stocks, 4) inbreeding resulting from the selection of unpedigreed siblings or half-siblings for broodstock, or any combination of these and other, unknown factors (Hedgecock and Sly 1990). They further stated that these kinds of management practices would not have been sufficient to maintain genetic variability in the long run, nor to prevent eventual inbreeding depression. In addition, significant inbreeding depression for yield and individual growth rate was observed in families with inbreeding coefficient of 6% in this species (Evans et al. 2004). To minimise the potential negative effects of inbreeding on larval and adult growth and survival, oyster hatcheries should use broodstock or a number of broodstock that could result in a maximum inbreeding coefficient of 0.25.

Table 7.7. Expected mortality rate (%) caused by inbreeding at different number of lethal equivalents per individual

Lethal equivalent	Inbreeding coefficient (%)		
	0.25 [1]	1.00 [2]	2.50 [3]
10	2.50	9.52	22.12
11	2.71	10.42	24.04
12	2.96	11.31	25.92
13	3.20	12.19	27.75
14	3.44	13.06	29.53
15	3.68	13.93	31.27
16	3.92	14.79	32.97
17	4.16	15.63	34.62
18	4.40	16.47	36.24
19	4.64	17.30	37.81
20	4.88	18.13	39.35

[1] This level of inbreeding is equal to the progenies produced by 50 males and 50 females with equal genetic contributions.
[2] This level of inbreeding is equal to the progenies produced by 25 males and 25 females with equal genetic contributions.
[3] This level of inbreeding is equal to the progenies produced by 10 males and 10 females with equal genetic contributions.

7.4. Concluding remarks

Results from the experiments in this chapter reveal that:

- The genetic load of Pacific oyster is high, ranging from 11.0 to 24.5 relative to pooled eggs controls, or 7.5 to 21.1 relative to pooled fresh sperm controls.
- The lethal equivalents (genetic loads) in Pacific oysters had been expressed progressively with larval development.

- Inbreeding depression in larval shell length was highly variable between individuals, with no inbreeding depression in 2 selfing families whereas it was significant at day 20 post-fertilisation in the other 2 families.
- Larval shell lengths differed significantly between different selfed families at day 20 post-fertilisation.
- In 2 of the 4 selfing groups, the larval shell lengths in pooled eggs controls were significantly larger than pooled fresh sperm controls at day 20 post-fertilisation.
- The sperm from different individuals survived the extended one year liquid nitrogen storage differently, with the highest fertilisation rate being 60%.
- Self fertilisation employing cryopreserved sperm is a feasible method that can be used in Pacific oyster breeding program to preserve genetic materials and to develop inbred lines and exploit hybrid vigors.
- To minimise the potential negative inbreeding effects on larval and adult growth and survival for commercial productions, oyster hatcheries should select or use broodstock with a clear breeding history to avoid mating relatives.
- At about one year post-fertilisation, the gonad of selfed progenies larger than 40 mm in length was well developed and could be sexed.
- The methods used for induction of oyster metamorphosis in commercial hatcheries were not suitable for our proposed experiments in which maintaining all sizes of larvae are required.

8. PRODUCTION OF HOMOZYGOUS (DOUBLED HAPLOID) OYSTERS

8.1. Introduction

Hybrid vigor refers to the phenomenon in which hybrid progeny of diverse inbred varieties exhibit greater biomass, speed of development, and fertility than the better of the two parents (Birchler et al. 2003). In maize breeding, the substantially higher yield is one of the reasons why hybrids were so rapidly accepted by the maize growers. Another undoubted factor is the greater uniformity of hybrids, which is useful for machine harvesting. Possibly the most important reason for the rapid spread in the 1930s was that the hybrid strains were strikingly more resistant to drought than the open-pollinated varieties in use (Crow 1998). In addition, the yields have continued to increase, with no sign that the rate of increase is diminishing after more than 60 years exploitation of hybrid vigor in maize (Crow 1998).

Challenges in developing a breeding strategy to exploit hybrid vigor are relevant for practical applications, which depend on the performance of the crosses as well as the cost-effective production and maintenance of the inbred lines. In fish, low genetic loads (less than 2) have been found in two species studied so far (McCune et al. 2002), and clones have been established in approximately 20 species (Komen and Thorgaard 2007). Doubled haploids have been used efficiently in selective breeding to estimate heritability of the traits of interest and QTL mapping (Zimmerman et al 2005). However, high degrees of heterosis, compatible with that found for crops, have not been reported in fish species yet (Wohlfarth 1993, Bryden et al. 2004), although the biggest challenge still is the extremely low yields of doubled haploids.

Heterosis for growth and survival in Pacific oysters *C. gigas* was first demonstrated experimentally by Hedgecock et al. in 1995 and 1996. High degrees of heterosis were confirmed later by Hedgecock and Davis (2007). Their result also showed that both additive and non-additive components of variance are important contributors to oyster yield. Yield generally increased with general combining abilities (GCA), as expected, but high-yielding hybrids with high, positive specific combining abilities (SCA) and little GCA were also observed. The non-additive genetic component of yield variance

is often the largest (Hedgecock and Davis 2007). These findings suggest that improvement of commercial oyster production could be achieved by a combination of selection among inbred lines and selection for SCA (Hedgecock and Davis 2007). The only commercial-scale trial showed that the cross used was better performing than current industry stock, even though that cross was clearly inferior to several other hybrid combinations used in their study (Hedgecock and Davis 2007). The inbred families used in their diallel crosses were mainly second-generation full-sib mated lines with inbreeding coefficients of $F = 0.375$. The diallel cross is a mating design resulting from the crossing of n parents in all possible n^2 combinations.

Theoretically, the doubled haploid technique is the most efficient method to establish pure inbred lines, and requires two generations (one generation for production of doubled haploid founders and another generation for replication of individual founders into identical inbreeding lines). The second most efficient method is selfing with an inbreeding coefficient of $F = 0.5$ per generation. Simultaneous hermaphroditism is very rare in Pacific oysters and can not be used as a routine technique. Given the rate of sex change from male to female in the subsequent spawning season found in this study (17.6%), self-fertilisation using cryopreserved sperm would be a feasible method. However, in comparison with doubled haploid method, more generations (> 6) will be needed to achieve a high inbreeding level. Sib-mating ($F = 0.25$) and gynogenetic techniques of inhibiting the 1st or 2nd polar body formation (Lines B and C in Figure 8.1; $F = 0.25$-0.50) are a method resulting in an even milder inbreeding coefficient per generation. Polar body II gynogenesis should be more efficient than polar body I gynogenesis for achieving a rapid accumulation of inbreeding.

Gynogensis is female parthenogenesis in which the embryo contains only maternal chromosomes, because the fusion between nuclei from egg and sperm failed. Gynogensis can be achieved by fertilizing normal eggs with sperm genetically inactivated by UV irradiation. In bivalves, however, resulting haploid embryos need to be restored to diploid to survive beyond the D-larval stage. Theoretically, the restoration of diploid status can be achieved by blocking the 1st polar body formation at the meiotic 1 division (Figure 8.1B), the 2nd polar body formation at the meiotic 2 division (Figure 8.1C) or the 1st mitotic division (Figure 8.2B). As in plant breeding,

the progeny produced by inhibiting the first mitotic division are called doubled haploid. It should be noted that doubled haploids can also be produced by androgenesis, where the haploid chromosomes doubled at the fist mitotic division originate from sperm (male), while the female genetic materials are denatured prior to fertilisation.

In Pacific oysters, gynogenetic diploids have been successfully produced by inhibiting the 2nd polar body formation (Guo et al. 1993b, Li and Kijima 2006). The high level of residual heterozygosity (due to a high recombination rate) found in their studies suggests that meiotic gynogenesis is not an effective method for rapid inbreeding in this species. However, it would still be one of the ideal methods for establishing doubled haploid lines, if doubled haploid founders can be produced initially.

The aim of the study was to optimize or develop a technique to produce doubled haploid Pacific oyster founders by inhibiting the 1st mitotic division of eggs fertilized with the sperm genetically inactivated by UV irradiation.

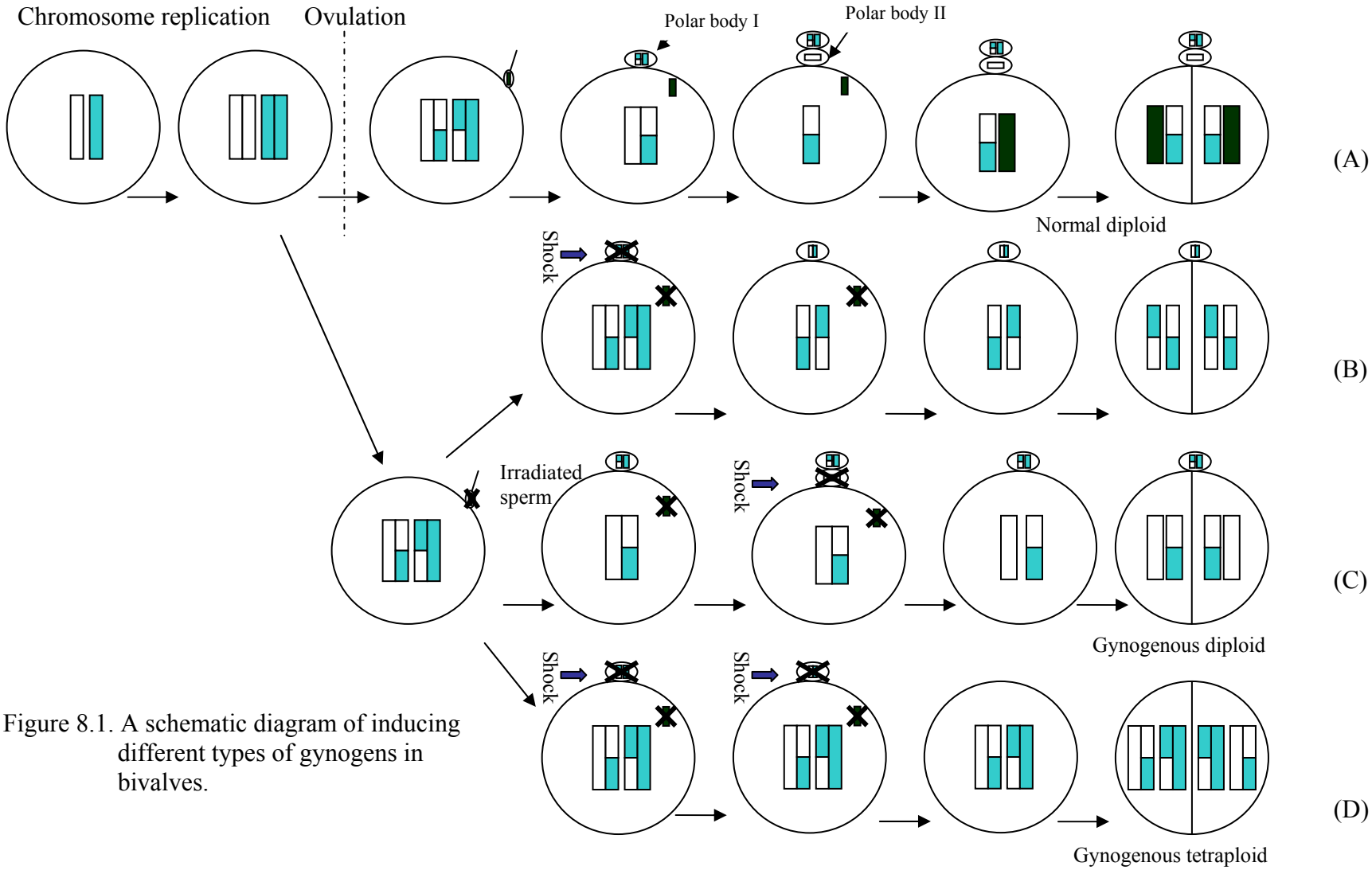

Figure 8.1. A schematic diagram of inducing different types of gynogens in bivalves.

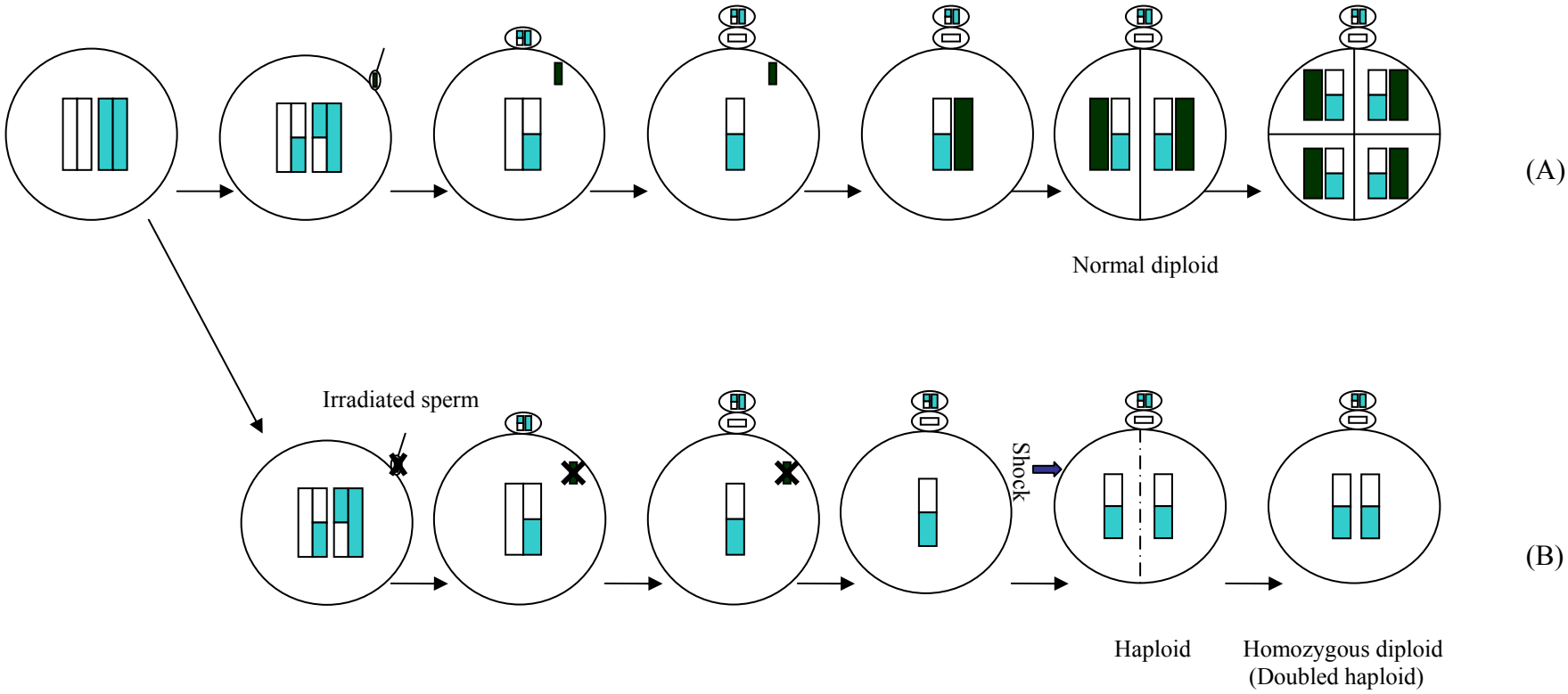

Figure 8.2. A schematic diagram of inducing different types of gynogens in bivalves.

8.2. Materials and methods

8.2.1. Experimental design

8.2.1.1. Gamete collection

The strip spawning methods described in chapter 7 (Genetic load of Pacific oysters farmed in Australia) were also applied in this study. Eggs from each female broodstock were kept separately and handled with a separate set of utensils in the subsequent experiment. Sperm from at least 3 males were pooled. After counting under the microscope with diluted subsamples, the required sperm concentration was achieved by adding 0.45 μm filtered seawater. Fertilisation, treatment and embryo culture were all conducted at 25^{o}C.

8.2.1.2. UV irradiation of sperm

The optimal UV dosage for denaturing Pacific oyster sperm DNA was determined by exposing 3mL of sperm at a concentration of 125 ~ 200 X 10^6 sperm/mL to 8 Watts UV light (VUC-18, ULTRA LUM, Inc. CA, USA) for 0, 1, 2, 3, 4 or 5 min. A subsample of 0.1 mL for each treatment was used to fertilise 200,000 eggs pooled from at least 5 females. Three lots of 200,000 eggs that were not fertilised were used as negative controls.

The method described by Guo et al. 1993 for UV irradiation of Pacific oyster sperm was also applied in this study. Briefly, the UV lamp was turned on at least 10 min prior to irradiation. The sperm suspension was then placed underneath the UV light at a distance of 10 cm from the lamp centre. The suspension was 1 mm in depth and held in a Petri dish of 8.5 cm in diameter. During the irradiation the Petri dish was shaken at least once every second.

After treatment, eggs fertilised with sperm irradiated for different durations were stocked in 20 L tanks separately, with a resultant density of 10 individuals/mL. The

activated eggs were determined as those eggs that had released their polar body(ies) or cleaved at 90 min post-fertilisation. Eggs survival to the D-larval stage were determined for each treatment at 24 h post-fertilisation.

8.2.1.3. Fertilisation and production of doubled haploids

After 4 min of UV irradiation, a 3 mL sperm suspension was used to activate strip-spawned eggs from one female broodstock. The activated eggs were then divided into two groups. The first group was a haploid control, where less than 20,000 activated eggs were allowed to develop without the blockage of first mitotic division. The second group was the doubled haploid group, where the remaining activated eggs (8 to 19 million per individual) were treated by one of the following methods to prevent the first mitotic division.

From 12th February 2004 onward, UV irradiation of 3.5 min duration was used because the 4 min treatment resulted in a maximum of 23% fertilisation rate in the last 5 treatments (families 31 to 35).

1) The combined hydraulic pressure technique

   This technique was developed by the author in previous projects and has been trialed in this study. The pressure equipment and the stainless steel pressurizing chambers were all custom-built. To double the chromosome set for generating doubled haploid progenies, the combined hydraulic treatment was applied 49 min post-fertilisation at 25^{o}C. For details, refer to the accompanying confidential document.

2) The cytochalsin B (CB) treatment

   The method described by Li (2007) for mitotic tetraploid inductions were also applied in this study at the first mitotic division to resume the diploid status of the eggs activated by UV irradiated sperm. Briefly, after embryos had been incubated in 0.5mg/L CB in 0.05% dimethylsulfate (DMSO) for 20 min from 50 min post-fertilisation at 25^{o}C, they were rinsed in 0.05% DMSO for another 20 min.

Due to very high workload during the experiment period and no D-larval development with the UV irradiation duration used in the UV irradiation of sperm experiments, the negative haploid controls were established at random at each cycle of doubled haploid production, or when the treatment method was changed.

The haploid controls were stocked in 5 L containers at a concentration of 4 eggs or larvae/mL. The embryos in the doubled haploid groups were reared in conical tanks (Figure 7.1) and stocked at an initial density of approximately 200 eggs/mL or 4 million eggs per tank. Therefore 2 to 5 tanks were required for each doubled haploid family for the initial 3 to 4 days. The larval density was kept at < 20 individuals/mL thereafter, and tanks were maintained according to the methods described in the section *7.2.1.3. Larval rearing*.

Methods used in chapter 7 in the previous summer were further improved to address the following two issues:

1. Heavy mortalities and low metamorphosis rates experienced among larvae when treated with epinephrine to induce metamorphosis and returned to the tank where the untreated larvae of the same group (family) were held. Returning treated larvae to the larval tank first and then screening the metamorphosed oysters the following day is the standard method used in commercial oyster hatcheries.

2. The water flow rates to the spat bottles could not be adjusted according to the requirements to suspend spat of different sizes. If the system was adjusted according to the requirement of large (or older) spat the system either lost the small or newly metamorphosed spat, or the screen on the outlet was blocked by the small spat. If the system was adjusted according to the small spat, the large spat crowded on the bottom of the bottle.

The first issue was addressed by the establishment of a new small metamorphosis system (Figure 8.3). With this system, many batches (1~30) of small quantities of larvae can be induced to metamorphose simultaneously. After epinephrine treatment the newly metamorphosed larvae can be held in this system for one day prior to being transferred into the spat bottle directly. The second issue was addressed by adding a

236 µm screen in the middle of the spat bottle to block the escape of newly metamorphosed larvae when the water flow was adjusted to suspend the larger spat (Figure 8.4).

Figure 8.3. New oyster metamorphosis system.

Figure 8.4. Modified spat rearing bottles. The arrow indicates the section modified. (For existing spat rearing bottles refer to Figure 7.2)

8.2.1.3. Microsatellite analysis

Female parental DNA was individually extracted from frozen muscle tissues using a salt extraction method (Miller et al. 1988) and the oyster larvae were separated under the microscope and their DNA was individually extracted using the Flinders Technology Associates (FTA®) paper method (Smith and Burgoyne 2004).

Six microsatellite loci were selected from 11 loci tested. They were Cg44, Cg49, Cg108 (Magoulas et al. 1998), L16 (Huvet et al. 2000), cmrCg02 and CgDT01 (Genbank). PCR amplifications were performed under the following conditions: 1 cycle of 9 min at 94°C, 35 cycles of 45 sec at 92°C, 30 sec at the primer-specific annealing temperature (53°C for Cg108, cmrCg02, and CgDT01 and up to 58°C for Cg44, Cg49 and L16) and 10 sec at 72°C, followed by a final cooling step of 10 sec at 30°C using Hybaid Omn-E thermal cycler. A "touchdown" protocol was used for all loci, dropping down over 15 cycles from 65°C.

Reaction mixes of 15µL volume contained 20 ng template DNA, 2.5 mM $MgCl_2$, 0.125 mM of each nucleotide, 0.5 µM of each primer [forward primer fluorescently labelled 6-FAM (fluorescein; GeneWorks) for Cg49 and CgDT01, NET (ABI PRISM BigDye primers, Apllied Biosystems) for L16 and cmrCg01, and VIC (ABI PRISM BigDye primers, Applied Biosystems) for Cg108 and Cg44], 1.5 µL GeneAmp 10X PCR Gold buffer and 0.06 µL of AmpliTaq Gold DNA polymerase (Applied Biosystems). For each individual, the PCR products of 2 to 3 loci were mixed (multiplex) and run on an ABI PRISM 3700 DNA analyser using ABI PRISM POP-6 polymer gels (Applied Biosystems). Product sizes were determined using the software ABI PRISM GeneScan and ABI Prism Genotyper version 3.7 (Applied Biosystems).

8.3. Results and discussion

8.3.1. UV irradiation of sperm

Techniques on denaturing the sperm DNA with UV light treatment have been tested by using the gametes collected from conditioned broodstock. The results (Figure 8.5)

showed that the sperm's ability to activate eggs was reduced from more that 95% in the control group (untreated sperm) to about 70% when sperm irradiated by UV light for 4 min. This ability was further reduced to less than 45% when sperm was irradiated for 5 min. The percentage of zygotes that developed into D-stage larvae was 0 in the 4 and 5 min UV treatment groups and 0.01% ~ 0.02% in the 2 and 3 min UV treatment groups. In the control group, 49% of the zygotes developed into D-stage larvae. These results indicate that the sperm chromosomes were successfully inactivated by the UV irradiation.

Published data (Guo et al. 1993b, Li et al. 2000) and our unpublished information show that the UV treatment duration, which starts to significantly reduce the percentage of egg activation, is normally considered as the optimal UV dosage for destruction of sperm chromosomes in Pacific oysters. Therefore, 4 min or slightly longer would be the optimal irradiation duration with the equipment used in this study.

In this study, UV intensity was not measured. Therefore, the effects of UV intensity on treatment duration could not be compared directly with other published outcomes (Guo et al. 1993b, Li et al. 2000b). In this species the optimal UV intensity and exposure duration revealed by Li et al. (2000b) were lower and shorter than the thresholds suggested by Guo et al. (1993b). These findings suggest that, in addition to UV intensity, the optimal treatment thresholds would also depend on other factors such as UV sources.

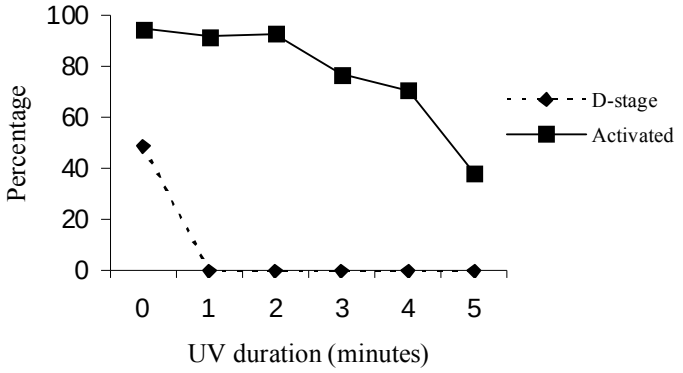

Figure 8.5. Percentage of eggs developed into D-larvae after being activated by sperm that were exposed to UV irradiation for different durations.

In these experiments 3 mL of sperm at a concentration of 125 ~ 200 X 10^6 sperm/mL was used for UV treatment each time. It was expected that 3 mL of sperm at these concentrations should be enough to fertilise the eggs (at least 10 million) from one female Pacific oyster of 10 cm in size (at a sperm/egg ratio of 12.5 ~ 20).

8.3.1. Production of doubled haploid progenies

The result from microsatellite analysis for one of the families produced by the combined hydraulic method with sperm irradiated for 4 min is presented in Table 8.1. Four larvae were homozygous on all the amplified loci (4 to 5), and the female broodstock were heterozygous at all these loci. This suggests that about 40% of the larvae produced in this family would be "pure" homozygous.

Table 8.1. Homozygosity of 15 day old larvae in family 9 produced by the combined hydraulic pressure technique and 4 min UV irradiation of sperm

Individual	Marker and allele[1]					
	Cg49	*CgTD01*	*Cg108*	*Cg44*	*L16*	*cmrCg02*
Mother 1	A B	A_1 B_1	A_2 B_2	A_3 B_3	A_4 B_4	A_5 B_5
Larva 1*	A A	x	B_2 B_2	B_3 B_3	A_4 A_4	x
Larva 2	x	x	x	A_3 A_3	A_4 B_4	A_5 A_5
Larva 3*	B B	x	x	A_3 A_3	A_4 A_4	B_5 B_5
Larva 4*	A A	B_1 B_1	x	B_3 B_3	A_4 A_4	A_5 A_5
Larva 5	A A	A_1 A_1	x	A_3 B_3	A_4 B_4	x
Larva 6	A A	A_1 A_1	x	A_3 A_3	A_4 B_4	A_5 A_5
Larva 7*	A A	A_1 A_1	A_2 A_2	A_3 A_3	x	x
Larva 8	B B	A_1 A_1	A_2 A_2	B_3 B_3	A_4 A_4	$\underline{C}$ B_5
Larva 9	A B	A_1 B_1	A_2 B_2	A_3 B_3	A_4 B_4	x

* Homozygous larvae; [1] Letters with underline represents a non-mother origin alleles; x = no result.

The number of loci that have been used to assess homozygous status of doubled haploids in fish species are highly variable, from 1 with recombination rate of 1 (Muller-Belecke and Horstgen-Schwark 1995, 2000) for gynogeneous doubled haploids or 1 with known heterozygous status in the donor male for androgeneous doubled haploids (Babiak et al. 2002) to as many as 11 (Castro et al. 2003). To determine the number of markers required for doubled haploid assessment, an understanding of potential contamination sources and the nature of the markers selected would be critical. As shown in Figures 8.1 and 8.2, the procedure used to produce doubled haploid progenies can also produce gynogenous diploids and tetraploids if other meiotic division(s) are prevented (Figure 8.1). However, the chance for the first meiotic division to be targeted is unlikely because this division will be completed within 25 min post fertilisation at 25^{o}C (Li 2007). Therefore, the main sources of contamination would be the paternal contribution through sperm that have escaped the UV treatment, or paternal residual transmission, or meiotic II

gynogens. If eggs were fertilised by sperm that have escaped UV treatment, normal zygotes will be produced. However, when the first mitotic division of these zygotes is inhibited by the doubled haploid treatment, the resultant progenies would be tetraploid. So far no mitotic tetraploid Pacific oyster progenies have survived beyond the D-larval stages (Guo et al. 1994, Li 2007).

In the study on microsatellite genetic markers assessment of gynogenetic status in turbot (*Scophthalmus maximus*), Castro et al (2003), using the CERVUS 2.0 package, assessed the power of the exclusion probabilities at each locus and the combined probability over loci to exclude a candidate parent from a single individual either knowing only the genotype of this offspring or also knowing the genotype of one parent, and suggested that in practice it would be sufficient to analyse only 2 carefully selected diagnostic loci in the offspring to confirm exclusive maternal contribution, provided the genotype of the mother was known (the normal situation in induced gynogenesis) and the variability of the markers were higher. They stated that the selection of 2 loci had also taken into consideration the mutation rate and the possible existence of paternal residual transmission in some offspring (Thorgaard et al. 1985). The number of alleles per locus in Castro's et al. (2003) study was similar to the variations of microsatellite markers found in Pacific oysters by Li and Kijima 2006 and in this study during the marker evaluation and selection stage. By chance three of the 6 microsatellite loci (L16, Cg 49 and Cg108) used in this study were also applied by Li and Kijima in 2006 (8 loci in total) to confirm the maternal origins of progenies in meiotic II gynogenetic Pacific oyster families.

The probability (***P***) for differentiating meiotic II gynogens from doubled haploids with different number of genetic markers can be calculated with the following equation:

$$\boldsymbol{P} = (1-(1-\mathbf{R}_1)\,(1-\mathbf{R}_2)\,(1-\mathbf{R}_3)\,(1-\mathbf{R}_4)\,\ldots\,(1-\mathbf{R}_n))^* 100$$

where **R** is the recombination rate of the genetic marker used. It can be further simplified if the mean recombination rate (**$\underline{\mathbf{R}}$**) is used:

$$\boldsymbol{P} = (1-(1-\underline{\mathbf{R}})^n)^* 100$$

Both equations assume that the markers used were not linked. The equations show that the higher the recombination rate the less the number of loci would be required for the proposed differentiations. In the extreme situation, if a marker has a recombination rate of 1 (100%), it is good enough for proper differentiation between meiotic II and mitotic gynogenetic origins. The application of this kind of assessment for the confirmation of the first generation DH progeny is based on the fact that the meiotic II gynogens are the product of meiotic non-disjunction; all pairs of homologous chromosomes originate from sister chromatids and are genetically identical prior to recombination events during the first meiotic division. While the mitotic gynogens are the product of mitotic non-disjunction, all pairs of chromosomes originate from identical sister chromatids. For example, the recombination rate of adenosine deaminase alloenzyme marker was found to be 1 in tilapia (*Oreochromis niloticus*), and this marker has allowed the proper differentiation between doubled haploid and meiotic II gynogenetic progenies in that species (Muller-Belecke and Horstgen-Schwark 2000). In the present study an average value of 0.74 was used at the time for microsatellite marker selection in 2003. This recombination rate was reported by Guo and Gaffney (1993) basing on allozyme markers, which is slightly higher than the average level (0.72) reported recently by Li and Kijima (2006) using microsatellites. According to the above equation, if the average recombination rate is 0.74, four or five unlinked markers would provide a confidence of 99.5% or 99.9% (99.4% or 99.8% if the rate is 0.72) to detect meiotic II gynogenetic progenies in doubled haploid productions in Pacific oysters, which would be reasonable for the present study.

Table 8.2. Homozygosity of the 15 day old larvae in the family 37 produced by the combined hydraulic pressure technique and 3.5 min UV irradiation of sperm

Individual	Marker and allele[1]					
	Cg49	*CgTD01*	*Cg108*	*Cg44*	*L16*	*cmrCg02*
Mother 2	X B	X_1 X_1	x	x	X_4 Y_4	X_5 B_5
Larva 1	X D	x	x	x	A_4 Y_4	A_5 X_5
Larva 2	X E	F X_1	x	x	X_4 Y_4	G_5 X_5
Larva 3	B B	x	x	x	X_4 Y_4	B_5 B_5
Larva 4	X D	x	x	x	A_4 Y_4	A_5 X_5

* Homozygous larvae; [1] Letters with underline represents a non-mother origin alleles; x = no result.

All larvae in Table 8.2 were heterozygous. They were produced by the same method as those in Table 8.1, except the sperm used were irradiated for 0.5 min less. These results suggest the 3.5 min irradiation duration was not strong enough to properly denature the sperm DNA. As expected, it was not possible to determine if the non-mother alleles originated from sperm escaped the UV treatment or from the paternal residual transmissions. It should be noted that when the project started in July 2002, less than 40 microsatellites were available from Genbank and literature for Pacific oysters, and have not been linkage-grouped. Therefore the markers used in this study were randomly selected. However, the 4 loci found in Hubert and Hedgecock's (2004) microsatellite DNA marker linkage maps for this species showed they are in different linkage groups (Cg49 in linkage group III, Cg108 in group VI, L16 in group VII and Cg44 in I), suggesting the markers selected in this study should have good chromosome representations. It is not clear why some markers had low expression rate in this study (Cg108 and Cg44 in Table 8.2). It might be due to the mixture of PCR products of different loci (multiplex) (Q Li, personal communication).

Table 8.3. Experimental results from different doubled haploid induction methods trialed in this study

Treatments	Fertilisation date	Family ID	No. of eggs per female (x 10^6)	Fertilisation rate (%) [1]	No. of D-larvae [2] (3-4 day old)	No. of 20 day old larvae [3]	No. of young spat
Combined hydraulic pressure							
4 min UV sperm irradiation							
	24/12/2003	Haploid 1*	0.02		0		
		1	10.0	60.0 ± 3.2	27,000	2,500	1
		2	10.0	47.0 ± 3.2	6,000	500	2
		3	25.0	29.3 ± 0.9	3,000 (discontinued)		
		4	19.0	30.0 ± 1.5	5,000 (discontinued)		
	26/12/2003	Haploid 2	0.02		0		
		5	10.0	44.3 ± 2.9	18,000	1,000	20
		Haploid 3	0.02		0		
		6	10.0	52.3 ± 4.9	6,600	1,000	18
		7	10.0	55.0 ± 4.5	1,000 (discontinued)		
		8	10.0	4.3 ± 0.7			

			(discontinued)			
	9	12.0	33.3 ± 1.7	122,000	7,000	5
	10	13.0	62.7 ± 1.8	87,000	3,000	9
	11	12.0	8.0 ± 1.0 (discontinued)			
	12	0.9	50.3 ± 1.5	30,000	2,200	1
	13	12.0	50.3 ± 0.9	73,000	2,000	12
	14	12.0	47.7 ± 1.5	154,000	3,500	4
30/12/2003	15	10.0	46.3 ± 0.9	80,000	2,000	3
	16	10.0	28.0 ± 1.7	44,600	1,000	2
	17	10.0	47.7 ± 1.5	13,500	6,000	3
2/01/2004	18	12.0	70.7 ± 0.7	17,200	800 (lost)	
	19	12.0	63.0 ± 2.6	71,500	3,000	39
	20	12.0	11.0 ± 1.0 (discontinued)			
	21	12.0	66.0 ± 3.1	40,000 (discontinued/ contamination)		
5/01/2004	22	12.0	57.0 ± 3.5	3,400 (discontinued)		
	23	12.0	53.3 ± 0.7	96,800	5,700	0

		24	12.0	30.3 ± 2.0	52,000	3,600	1
		25	12.0	42.3 ± 2.2	44,000	3,800	1
	7/01/2004	26	10.0	49.3 ± 1.3	130,000	8,000	6
		27	10.0	57.7 ± 3.2	21,000	8,800	13
		28	10.0	39.0 ± 1.5	120,000	9,000	3
	2/02/2004	Haploid 4	0.02		0		
		29	0.9	8.0 ± 0.6	1,000 (discontinued)		
	5/02/2004	30	11.0	4.0 ± 0.6	900 (discontinued)		
	6/02/2004	31	10.0	22.7 ± 1.2	800 (discontinued)		
	9/02/2004	32	12.0	19.7 ± 1.8	600 (discontinued)		
	10/02/2004	33	8.0	10.3 ± 0.9	1,000 (discontinued)		
Combined hydraulic pressure							
3.5 min UV sperm irradiation							
	12/02/2004	Haploid 5	0.02		25		
		36	10.0	49.0 ± 5.5	800 (discontinued)		
		Haploid 6	0.02		112		
		37	12.0	74.0 ± 2.9	8,000	(discontinued)	

		38	10.0	38.0 ± 1.7	200 (discontinued)	
	13/02/2004	39	15.0	72.3 ± 0.3	2,500 (discontinued)	
		40	10.0	43.3 ± 3.5	3,600 (discontinued)	
		41	16.0	49.7 ± 2.9	20,000	(discontinued)
		42	13.0	45.7 ± 2.9	5,000 (discontinued)	
	17/02/2004	43	14.0	56.0 ± 1.2	25,000	(discontinued)
		44	9.0	48.3 ± 1.8	20,000	(discontinued)
		45	19.0	52.0 ± 1.0	11,000	(discontinued)
	18/02/2004	46	6.5	40.0 ± 1.5	3,000 (discontinued)	
		47	13.4	57.3 ± 2.4	30,000	(discontinued)
		48	12.1	52.7 ± 1.2	5,000 (discontinued)	
		49	12.8	52.7 ± 0.9	3,000 (discontinued)	
Cytochalasin B (0.5 mg/L) ***4 min UV sperm irradiation***						
	8/01/2004	34	10.0	53.3 ± 1.2	150 (discontinued)	

	35	15.0	59.7 ± 0.3	230 (discontinued)

[1] Most families with less than 11% fertilisation rate were discontinued.
[2] Families with less than 5000 D-larvae were discontinued.
[3] Families produced with the method resulting in no homozygous larvae in the preliminary DNA analysis were discontinued.
[*] The haploid control was established using the same sperm and eggs as the next family but without the combined hydraulic pressure treatment.

Table 8.4. Percentage of fertilized eggs survived to different stages after doubled haploid treatment

Fertilisation date	Family ID	D-larvae (3-4 day old)	20 day old larvae	Young spat *
Combined hydraulic pressure				
4 min UV sperm irradiation				
24/12/2003	Haploid 1#	0		
	1	0.45000	0.04167	0.00002 (0.04)
	2	0.12766	0.01064	0.00004 (0.40)
	3	0.04091		
	4	0.08772		
26/12/2003	Haploid 2	0		
	5	0.40602	0.02256	0.00045 (2.00)
	Haploid 3	0		
	6	0.12611	0.01911	0.00034 (1.8)
	7	0.01818		
	9	3.05000	0.17500	0.00013 (0.07)
	10	1.06792	0.03682	0.00011 (0.30)
	12	6.62252	0.48565	0.00022 (0.05)
	13	1.20861	0.03311	0.00020 (0.60)
	14	2.69231	0.06119	0.00007 (0.11)
30/12/2003	15	1.72662	0.04317	0.00006 (0.15)
	16	1.59286	0.03571	0.00007 (0.02)
	17	0.28322	0.12587	0.00006 (0.05)

2/01/2004	18	0.20283	0.00943	
	19	0.94577	0.03968	0.00052 (1.3)
	21	0.50505		
5/01/2004	22	0.04971		
	23	1.51250	0.08906	0.0000 (0.00)
	24	1.42857	0.09890	0.00003 (0.03)
	25	0.86614	0.07480	0.00002 (0.03)
7/01/2004	26	2.63514	0.16216	0.00012 (0.08)
	27	0.36416	0.15260	0.00023 (0.15)
	28	3.07692	0.23077	0.00008 (0.03)
2/02/2004	Haploid 4	0		
	29	0.13889		
5/02/2004	30	0.20455		
6/02/2004	31	0.03529		
9/02/2004	32	0.02542		
10/02/2004	33	0.12097		

Combined hydraulic pressure
3.5 min UV sperm irradiation

12/02/2004	Haploid 5	0.12500		
	36	0.01633		
	Haploid 6	0.56000		
	37	0.09009		
	38	0.00526		
13/02/2004	39	0.02304		
	40	0.08308		
	41	0.25168		
	42	0.08422		
17/02/2004	43	0.31888		

	44	0.45977
	45	0.11134
18/02/2004	46	0.10033
	47	0.38933
	48	0.07833
	49	0.04457
Cytochalasin B (0.5 mg/L)		
4 min UV sperm irradiation		
8/01/2004	34	0.00281
	35	0.00257

* Figures in brackets are the percentage of 20 day old larvae surviving to young spat.

\# The haploid control was established using the same sperm and eggs as the next family but without the combined hydraulic pressure treatment.

In the treatments conducted prior to 10th February 2004 (4 min sperm UV irradiation and CB or combined hydraulic pressure treatment to block the first mitotic division), no D-shaped larvae occurred in the four randomly established haploid controls, whereas 150 (CB) or 600 (combined hydraulic pressure) to 154,000 larvae were observed 3 to 4 days post-fertilisation in all the families treated to prevent the first mitotic division (Tables 8.3 and 8.4). These results suggest that progenies in the families treated at the first mitotic division were gynogens. Li et al. 2000a confirmed these gynogens were diploids in a similar study on this species. The ploidy levels of the resultant D-shaped larvae were not assessed in this study. However, the application time (49 or 50 min post-fertilisation) and duration (≤ 20 min) of the CB or combined hydraulic pressure treatment suggested only one cell division would be affected at 25°C (Li 2007). Preliminary microsatellite markers indicated they were meiotic or mitotic (doubled haploid) gynogens (Table 8.1). In comparison with the percentages of D-shaped larvae produced by the combined hydraulic pressure treatment (average 1.244%), the percentages of D-shaped larvae produced by CB treatment were very low (less than 0.003%). Extremely low resultant D-shaped larvae were also experienced by Li et al. (2000a) in their attempt to produce doubled haploid Pacific oysters by the method similar to the CB treatment used in this study. However,

the homozygous status of resultant progenies was not evaluated in their study. Therefore, the 15 day old homozygous Pacific oyster larvae produced by the combined hydraulic pressure method in this study are a novelty among molluscs.

Pacific oysters farmed in Coffin Bay, South Australia spawned naturally in late December 2003. The broodstock used in this study were originally from this region and their spawning had been prevented at 22^{o}C or less at SARDI. However, the quality of sperm stripped from them deteriorated substantially between 2nd and 10th February 2004, resulting in low fertilisation rates (4.0% to 22.7% with an average of 12.9%) after 4 min UV irradiation duration (families 31 to 35, Tables 8.3 and 8.4). The fertilisation rate increased to the levels prior to 17th January 2004 (average 52.4%) when the duration of UV irradiation on sperm was reduced to 3.5 min (an average of 52.6% from families 36 to 49, Tables 8.3 and 8.4). Reduction in sperm irradiation duration also resulted in the appearance of D-shaped larvae in the haploid controls (Haploids 5 and 6), suggesting that nucleic materials in some sperm had not been properly denatured. The preliminary microsatellite DNA marker analyses also supported this explanation, because new alleles (non female originated alleles) were detected in the larvae assessed (Table 8.2). It should be noted that these results were from a small sample size and old larvae (15 day old). A higher proportion of homozygous larvae would have already died prior to this age, due to the very high genetic load in this species (for details refer to previous chapter).

The results from the families produced using sperm irradiated for 3.5 min further confirmed that in addition to the treatment methods used, gamete quality is another critical parameter determining the success of gyogenetic inductions in Pacific oysters.

As predicted in the previous genetic load analyses, high mortalities were experienced after inhibiting the first mitotic division of the eggs activated by sperm irradiated by UV light for 4 min. The average rates of activated eggs surviving to 3 to 4 days and to 20 days post-fertilisation in the first 33 families were, respectively, 1.2435% (0.0182% to 6.623%) and 0.0974% (0.0094% to 0.4857%). These are within the ranges predicted in the previous genetic load study (for details refer to the preceding chapter). However, these survival rates would be biased by the potential side effects of gynogenetic treatments, which would theoretically reduce the survival rate, and the

resultant meiotic gynogenetic progenies, which would theoretically increase this rate. Both these potentially influential parameters could not be differentiated and/or determined in this study.

Issues of very high mortality during or after the metamorphic stage experienced in the genetic load study in the previous chapter have not been resolved through the modification of the existing system at SARDI (*8.2.1.3. Fertilisation and production of doubled haploids*). On average, only 0.41% (0.00% ~ 2.00%) of 20 day old larvae survived to the young spat stage, approximately 10 days after the last lot of larvae collected by the 236 μm screen was induced to metamorphose by epinephrine (Tables 8.3 and 8.4). The use of a newly established bucket metamorphic system minimised high mortalities in the larval tanks experienced in the previous genetic load experiments, and the modification of the upwelling spat bottles have prevented the escape of newly "metamorphosed" larvae from the system. However, the survival rates in the spat bottles were very low. For example, more than 4000 "metamorphosed" larvae in family 9 have been transferred into their spat bottle, but only 5 survived to young spat. In our previous experiment on the effects of cryopreserved sperm on the development of progenies in this species, more than 15% of 19 day old larvae developed into young spat even though the same metamorphic treatment was applied (Zhang 2004). Results from this and previous chapters indicate that the metamorphic method used for commercial production might not be suitable for managing stock with a high inbreeding level ($F \geq 0.5$). The "weak" or highly inbred oyster larvae might be very sensitive to epinephrine treatment.

In December 2004, the average size of the "doubled haploid" individuals was about 3.0 cm in length (n = 10 randomly selected oysters) after a few months grow-out on a lease. Therefore, the proposed tentacle/mantle biopsy sampling (and thus DNA analyses) could not be performed.

8.4. Concluding remarks

The results in this study show that:

- Four minutes UV irradiation was effective in denaturing the stripped spawning Pacific oyster sperm under the parameters (sperm density and UV source) used in this study.
- The first mitotic division of eggs activated by UV irradiated sperm have been successfully blocked using the combined hydraulic pressure method. Preliminary results from DNA analysis of nine, 15 day old larvae, from one of these families revealed that 4 were homozygous on all the amplified loci (4 to 5), whereas the female broodstock used to produce these larvae were heterozygous at all these loci. This suggests that about 40% of the larvae produced in this family could be 'pure' homozygous with more than 99% confidence according to the average recombination rate of the markers (microsatellites and allozymes) published so far. During the 2003/2004 summer period in total 33 families (the first 33 families) have been produced using this experimental setting.
- After the combined pressure treatment the average rates of activated eggs surviving to 3 to 4 days and to 20 days post-fertilisation were low, being 1.2435% (0.0182% to 6.623%) and 0.0974% (0.0094% to 0.4857%), respectively. These rates were well within the ranges predicted in the genetic load study (details refer to chapter 7).
- Of the "doubled haploid" families produced by the above mentioned method, 19 reached the metamorphic stage (day 20 post-fertilisation), and the other 14 were discontinued due either to low survival rates or being lost by accident. The number of 20 day old larvae varied from 500 to 9,000 (4338 on average). High variation between families was expected based on the high variation in the number of genetic loads per individual revealed in the genetic load experiments.
- High mortalities were experienced during metamorphic period; the number of individuals per family decreased to an average of 7 (0 to 39). These results further confirm that the metamorphic method used for commercial production would not be suitable for managing stock with a high inbreeding level ($F \geq$

0.5). The "weak" or highly inbred oyster larvae might be very sensitive to epinephrine treatment.

In addition, the equations developed in the present study for estimating the number of genetic markers required to differentiate doubled haploid progenies from meiotic II gynogens would be critical for the commercial application of doubled haploid techniques, and could be applied to the similar assessment of other aquaculture species (with eggs being ovulated prior to the second meiotic division).

9. BENEFITS

This project is an initial feasibility study to build up a comprehensive breeding program for commercial production of genetically uniform batches of oysters with desired traits through the efficient and effective exploitation of additive and non-additive genetic variations. The main benefit from this project is the development of a method to produce doubled haploid oyster larvae, which is the first critical step towards the application of the doubled haploid technique in an oyster breeding program. The other benefits from this project to the oyster aquaculture industry can be described from the following two aspects:

1. The high genetic load revealed during the larval development period re-emphasised the importance of avoiding inbreeding in order to maintain consistent commercial hatchery productions. Inbreeding could be minimised by using a higher number of broodstock, and broodstock with recorded pedigree history to avoid mating relatives. The importance of this practice clearly must be recognised, especially when more and more growers farm selected family lines in Australia.

2. Sperm cryopreservation is a feasible method that can be used in off-season commercial hatchery production, family establishment and the preservation of genetic materials in Pacific oysters.

10. FURTHER DEVELOPMENT

This project aimed to investigate the method(s) to produce doubled haploids and related issues. Fifteen day old doubled haploid larvae have been induced using the combined hydraulic pressure method. However, heavy mortalities were experienced after epinephrine treatment to induce metamorphosis, and the remaining few progenies were small at the end of this study. Therefore, no information is available on their performance after metamorphosis and at maturity. Research on finfish species indicates that the fertility differs between male and female doubled haploids (Komen and Thorgaard 2007). The fertility of doubled haploid females is reduced in most species studied (Scheerer et al. 1991, Arai 2001), whereas the fertility in male doubled haploids is hardly affected at all (Santhakumar et al. 2003, Komen and Thorgaard 2007).

To further improve the doubled haploid techniques in this species, competent larvae need to be settled naturally so that the reasons for high mortality during this period can be investigated with either selfed or doubled haploid progenies. If it is caused by the expression of genetic load, the production of doubled haploid founders would be very difficult. Preliminary analysis indicates that if the levels of mortality experienced in this study were due to the expression of genetic load, at least another 12 lethal equivalents would be required. This means the average genetic load published for this species would be doubled, but this is highly unlikely.

If the doubled haploid larvae produced with the method used in this study could survive to maturation stage, it would provide a unique opportunity to investigate the sex determination mechanism in Pacific oysters. This information will, in turn, be used to determine the methods for establishing and maintaining the doubled haploid lines. It should be noted that the meiotic gynogenic method that is commonly used in fish species to maintain doubled haploid lines has already been established in Pacific oysters (Guo and Gaffney 1993; Guo et al. 1993).

Non destructive sampling methods for spat or young oysters would be required so that doubled haploids can be screened off at an earlier stage, which would minimise the workload and reduce the facility requirement for grow-out.

11. PLANNED OUTCOMES

This project aimed to build up the knowledge and techniques required to establish a comprehensive oyster breeding program using advantages of doubled haploids. The planned outcomes for this initial step include:

1. Knowledge of genetic load (or lethal equivalent) of Pacific oysters farmed in Australia

   In this study, the genetic load or the number of lethal equivalents in Pacific oysters was estimated using the relative larval survival rates of selfed families in comparison with one of the two outcrossed controls (pooled eggs control and pooled fresh sperm control). The pooled egg controls were produced by fertilising eggs pooled from 5 females (not used in the selfed families) with the sperm used for each of the selfed families. The pooled fresh sperm control was established by fertilising the eggs used in each of the selfed families with the fresh sperm pooled from 5 males of the same stock. The genetic load or the number of lethal equivalents per individual is high and expresses progressively with the development of Pacific oyster larvae, from an average of 3.7 on day 5 post-fertilisation to 15.0 on day 20 or from 5.1 to 11.5 according to the different controls used. The load expressed on day 20 was also highly variable between individuals, from 11.0 to 24.5 or 7.5 to 21.1. These results suggest that when doubled haploid progenies are produced, high and variable mortalities would be experienced during the larval period. Results from genetic load study also showed that the gonad of selfed progenies larger than 40 mm was well developed and could be sexed at their first maturation at approximately 12 months post-fertilisation.

2. Methods for doubled haploid production

   The first mitotic division of eggs activated by UV irradiated sperm have been successfully blocked by the combined hydraulic pressure method. Microsatellite marker analyses of 15 day old larvae showed that some of them were homozygous on all the amplified loci (4 to 5), whereas their female broodstock used to produce

these larvae were heterozygous at all these loci. This suggested that some of these larvae were 'pure' homozygous with more than 99% confidence, according to the average recombination rate of the markers (microsatellites and allozymes) published so far. During the 2003/2004 summer period, in total 33 families (the first 33 families) have been produced using this method. As predicted in the genetic load study, the resultant "doubled haploid" larvae surviving to day 20 post-fertilisation were low and highly variable between families, from 500 to 900,000. However, about 60% of the families kept produced 3,000 or more 20 day old larvae, which was reasonable in term of family size at this stage.

In addition, the equations developed in the present study for estimating the number of genetic markers required to differentiate doubled haploid progenies from meiotic II gynogens is critical for commercial application of doubled haploid techniques, and could be applied to similar assessment in other aquaculture species (with eggs being ovulated prior to the second meiotic division).

12. CONCLUSION

The following conclusions were drawn from the results of experiments conducted in this project.

1. Genetic load in Pacific oysters

   The genetic load or the number of lethal equivalents in Pacific oysters was high, highly variable, and expressed progressively with larval development. On day 20 post-fertilisation (one day prior to settlement induction), the average number of lethal equivalents per individual was 11.5 or 15.0, according to the two different outcross controls used.

2. Doubled haploid progeny production

   Fifteen day old doubled haploid Pacific oyster larvae were produced by the combined hydraulic pressure method and confirmed by microsatellite markers with 99% confidence. This provides the first evidence that doubled haploid bivalve larvae can survive to the stage close to metamorphosis.

3. Metamorphosis by epinephrine treatment

   Epinephrine treatment has been widely used for oyster metamorphosis to obtain single cultchless spat in commercial productions. However, very high mortalities were experienced in this study, suggesting that this method was not suitable for managing stock with a high level of inbreeding ($F \geq 0.5$). The "weak" or highly inbred oyster larvae might be very sensitive to such epinephrine treatment.

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APPENDIX 1: INTELLECTUAL PROPERTY

The combined hydraulic pressure method developed in author's previous projects has produced doubled haploid oyster larvae (analysed by microsatellite markers) in this study. This technique has the potential to substantially increase the efficiency and the effectiveness of molluscan breeding and genetic researches. For details refer to the accompanying confidential document.

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APPENDIX 3: FLOW CYTOMETRIC EVALUATION OF THE DEEP FREEZING DAMAGE TO PACIFIC OYSTER (*CRASSOSTREA GIGAS*) SPERM AND ITS CORRELATION TO SPERM FERTILITY

This chapter was submitted for publication as:

Zhang X, Li X, Li X, Yi Q. Flow cytometric evaluation of the deep freezing damage to Pacific oyster (Crassostrea gigas) sperm and its correlation to sperm fertility

Abstract

This study investigated the use of the FITC-PNA/PI and Rh123/PI assays to evaluate changes to Pacific oyster sperm quality after cryopreservation by flow cytometry, and its impact on the fertilization capacity of cryopreserved sperm.

The results from the FITC-PNA/PI assay showed that the percentage of normal acrosome sperm was significantly lower ($P < 0.05$) in cryopreserved sperm (15.80 ± 0.61%) than in fresh sperm (87.10 ± 1.57%). A similar result was found in the Rh123/PI assay on the sperm mitochondrial membrane. The percentage of sperm with intact mitochondrial membranes decreased from 91.27 ± 1.25% in fresh sperm to 19.73 ± 1.20% in cryopreserved sperm. Cryopreservation also led to a significant increase ($P < 0.05$) in the percentage of dead sperm and partially damaged or dying sperm. Our results also indicated that in both assays (Rh123/PI and FITC-PNA/PI assays) the percentage of live sperm after cryopreservation significantly positively correlated to fertility ($r = 0.901$, $P< 0.01$; $r = 0.802$, $P < 0.01$, respectively), while the percentage of partially damaged sperm significantly negatively correlated to fertility ($r = -0.871$, $P < 0.01$; $r = -0.884$, $P < 0.01$, respectively). Significant correlations were found between $Rh123^+/PI^-$ and $FITC\text{-}PNA^+/PI^-$ ($r = 0.829$, $P < 0.01$) and $Rh123^+/PI^+$ with $FITC\text{-}PNA^+/PI^+$ ($r = 0.940$, $P < 0.01$) results.

The results of the present study showed that cryopreservation caused extensive damage to the Pacific oyster sperm membrane, acrosome, mitochondria and chromatin structure, therefore affecting sperm fertility.

Keywords: Pacific oyster (*Crassostrea gigas*), sperm damage, flow cytometry, fertility, cryopreservation

A1. Introduction

Cryopreservation of Pacific oyster (*Crassostrea gigas*) sperm has been observed to result in the death of a considerable number of sperm (Kurokura, 1990; Zhang, 2004), and significant reduction in the percentage of fertilized eggs that develop into D-larvae at 48 hours post-fertilization (Zhang, 2004). In addition, the inconsistent performances of cryopreserved sperm or large variability in fertility of cryopreserved sperm are another issue for oyster sperm cryopreservation. Therefore, new methods are needed to increase the accuracy of predicting the fertilization potential of cryopreserved sperm in order to further improve the cryopreservation technique.

Many technical approaches for testing sperm function have been developed over the past 10-15 years with terrestrial species. Traditional methods mostly use microscope or visual observations, which are subjective and have low repeatability. Fluorescent staining improves microscopic evaluation of sperm, but its major drawback is that only 100 or 200 spermatozoa can be assessed per sample. Flow cytometry is potentially a powerful tool in the analysis of sperm function as large number of spermatozoa can be evaluated in a short time (Garner et al., 1986). In addition, flow cytometry can assess different aspects of sperm function simultaneously due to the application of combined fluorescent probes, such as *Pisum sativum* agglutinin (PSA) (Cross et al. 1986) or *Arachis hypogea* agglutinin (PNA) (Cheng *et al.*, 1996) labelled with fluorescein isothiocyanate (FITC) for acrosome injury and Rhodamine 123 (Rh123) (Graham et al. 1990) for mitochondrial activity.

To date, several methods to assess injury due to cryopreservation have been applied for thawed spermatozoa in some fish species. The following damages have been

reported: plasma membrane integrity (Labbe and Maisse, 1996), mitochondrial activity (Ogier *et al.* 1997), hypo-osmotic sensitivity (Malejac et al., 1990), sperm morphology (Lahnsteiner *et al*. 1996), sperm motility (Lahnsteiner *et al*. 1998) and DNA damage (Zilli et al., 2003; Gwo et al., 2003). However, studies on the cryopreservation of shellfish sperm have mostly been concentrated on the improvement of freezing techniques. Presently, there is no simple, accurate method of evaluating the quality and fertilizing capability of cryopreserved shellfish sperm, except for the visual estimation of sperm motility (Chao, 1996) and counts of fertilization rates for species in which eggs are readily available.

The objectives of the present study were to investigate if different functional parameters, acrosome and mitochondrial integrity of cryopreserved sperm from Pacific oysters, *Crassostrea gigas*, could be evaluated using fluorescent staining by flow cytometry and to determine their relationship with fertilization rate in order to identify those parameters that are best at predicting fertilizing potential in this species.

A2. Materials and methods

A2.1. Gamete collection

The brood stock used in this study was from Smoky Bay, South Australia and were transported to SARDI Aquatic Sciences Centre in Adelaide overnight in a refrigerated container. On arrival the shell surfaces were cleaned with a brush and then rinsed in fresh water before being put into an acclimatization tank on a flow-through system. The oysters were maintained at 18 °C in the tank for 2 to 6 days prior to being used in the experiments. During this period the animals were fed continuously with an algal mixture of *Isochrysis sp.* and *Pavlova sp*. On the day the experiments started a hole was drilled on the left shell of each oyster and a biopsy sample taken from the gonad using an 18-gauge needle. The sex of the animal was then determined by viewing the biopsy sample under a light microscope. For male oysters, only the individuals with sperm motility ratios ranging from 4 to 5 were selected for use in experiments (Legendre & Billard, 1980). To avoid cross-contamination between individuals one

syringe and needle was used for each oyster. After the top shell (right shell) was removed the gonadal contents were aspirated into a glass Pasteur pipette and squeezed into a glass beaker containing 0.45 μm filtered seawater.

Equivalent contributions of sperm from 5 oysters were pooled into a 100 ml beaker. The beaker was then left for 20 to 30 min at 18-20ºC. The debris was separated from the sperm by passing the suspension through a 20μm screen. The density of the sperm suspension was counted under a microscope using a hemocytometer after diluting the original sperm concentration 1000 times. The sperm concentration was then adjusted to 125×10^6/ml before use in experiments.

Eggs from 5 females were pooled into a 1000 ml glass beaker. The separation of gonadal tissues and other debris from the eggs was conducted by washing them through a 75 μm screen with a 20 μm screen underneath. The eggs were then washed into a new 1000 ml glass beaker with 0.45 μm filtered seawater and incubated at 24ºC for about 1 hour before being used in experiments.

A2.2. Spermatozoa cryopreservation

Spermatozoa cryopreservation was conducted according to the method described by Li et al. (in preparation). The concentration of cryoprotectants that resulted in the highest fertilization rates in the experiments conducted by Li et al. (in preparation) were also employed in this study (6% for DMSO and 0.6% for glycine). The stock solution of 12% DMSO (v/v) and 1.2% glycine was prepared freshly in 0.45 μm filtered seawater (salinity 37 ppt) and stored in a refrigerator. The sperm suspension was first diluted to a concentration twice that of the final concentration in the experiment and then mixed with the cryoprotectant stock solution at a 1:1 ratio, resulting in the correct concentration of chemical and sperm required in the experiments. Five pre-freezing sperm concentrations 62.5×10^6/ml, 50.0×10^6/ml, 37.5×10^6/ml, 25.0×10^6/ml and 12.5×10^6/ml were used in this study. The sperm cryoprotectant mixtures were mixed thoroughly by hand as soon as the sperm suspensions were pooled with the cryoprotectant solution. A Pasteur pipette was used to transfer 1.8 ml of the mixture into 2 ml cryovials with 6 replicates (cryovials) for

each sperm concentration. The cryovials were placed horizontally on a tray 10 cm above the surface of liquid nitrogen for 2 hours before being immersed into it. The cryovials were kept for at least 2 weeks in the liquid nitrogen (-196ºC) before being used in the following experiments.

A2.3. Fertilization

Prior to fertilization the eggs were collected on a 20 µm screen and washed into a 100 ml glass beaker using the minimum amount of 0.45 µm filtered seawater as possible. The concentration of the eggs was then calculated by averaging the results from three counts of 1 ml of egg solution that had been diluted 1000 times. The required egg concentrations were then produced by adding a calculated quantity of 24ºC freshly filtered seawater.

The cryovials were thawed by submersion in a 58-60ºC water bath and moved into a 20ºC seawater bath as soon as they had fully melted. The vials were kept in the water bath for 20-30 min before being used to fertilize any eggs.

Each of the three replicates in each experiment was produced by combining 3 ml of eggs with 3 ml of cryopreserved sperm. The sperm was subsampled from a pooled sample of three vials containing the same sperm concentration. Controls were established by fertilizing 3ml of eggs with 0.5ml of freshly collected sperm, 20×10^6/ml in density. All the crosses used in the experiments are described in Table 1.

At 4 and 8 min post-fertilization an equivalent amount of filtered seawater to that of the solution in the fertilization container was added. The fertilization container was then filled to 150 ml at 12 min post-fertilization. After 15 min fertilization, the exccess sperm was washed off the eggs. Eggs from each replicate were stocked in a conical bottomed 20 L polyester tank. Light aeration was provided from the bottom of the tank.

At 90 min post-fertilization the percent fertilization in each replicate was calculated by averaging the numbers of fertilized eggs in 100 oocytes in three random samples

counted under the microscope. Eggs that were at or beyond the first polar body formation or at a multiple cell stage were counted as fertilized eggs.

Table 1. The concentrations and type of the oyster sperm used in different groups

Groups[1]	Sperm type[2]	Sperm concentration ($\times 10^6$/ml)	Total sperm volume (ml)	Total egg number ($\times 10^5$)	Total egg volume (ml)	Sperm/ egg ratio
a	1	62.5	3	8	3	234.4
b	1	50.0	3	8	3	187.5
c	1	37.5	3	8	3	140.6
d	1	25.0	3	8	3	93.8
e	1	12.5	3	8	3	46.9
Control	2	20	0.5	8	3	12.5

[1] Three replicates in each experiment.

[2] 1: cryopreserved sperm; 2: fresh sperm.

A2.4. Evaluation of sperm quality by flow cytometry

Five min after thawing, the 3 extra vials in each group were diluted to a final sperm concentration of 3 - 5 x 10^6/ml with freezing medium. Then 2.8 ml was taken from each of the three diluted sperm solutions and mixed with 0.1 ml of fluorescent staining solutions. The staining solutions were made to a concentration that was 30 times higher than their required final concentrations. The samples were then incubated for the desired period prior to being assessed by flow cytometry.

A2.4.1. Acrosome integrity (FITC-PNA/PI assay)

In order to distinguish between different acrosomal conditions the acrosome was visualised by staining with a fluorochrome combination of FITC-conjugated peanut

agglutinin (*Arachis hypogaea*, PNA) and PI, according to the procedure of Cheng et al., (1996). Sperm samples containing FITC-PNA and PI at a concentration of 100 µl/ml and 5 µg/ml, respectively, were incubated in the dark for 30 min and then analysed by flow cytometry.

A2.4.2. Mitochondrial membrane potential (Rh123/PI assay)

To assess the proportion of sperm with high and low mitochondrial membrane potential, fluorescent staining with Rh123 and PI was carried out following the method of Graham et al. (1990). Briefly, sperm samples were incubated in the dark with Rh123 at a concentration of 5 µg/ml for 20 min. PI was added to the samples 5 min prior to flow cytometry analysis at a concentration of 5 µg/ml.

A2.4.3. Flow cytometry analysis

The samples were analyzed by a Becton Dickinson FACScan flow cytometer (BD Bioscience, SanJose, CA), equipped with an argon-ion laser that supplied 488 nm wavelength. Two light-scatters, forward scatter (FSC) and slide scatter (SSC), were used to define a gate that excluded debris and aggregates from all fluorescence analyses. PI fluorescence emissions were collected through a 670 nm filter (FL3); FITC-PNA and Rh123 fluorescence were measured through a 575/525 nm filter (FL1). A minimum of 10000 spermatozoa was examined for each sample. Flow cytometric dot plots for assays used in this study are shown in Figs. 2 and 4.

A2.5. Statistical analysis

Dot plots were drawn and flow cytometry-derived data were analysed using the software WinMDI 2.9 (http://facs.scripps.edu/software.html). The data were then exported to SPSS 10.0 for further statistical analyses. The differences in the percentage of events in the various quadrants between fresh and cryopreserved sperm were analysed using ANOVA. Pearson's correlation coefficients were applied to test the linear association between sperm functions resulting from the different assays and the fertility of cryopreserved sperm. Differences were considered statistically significant at $P < 0.05$.

A3. Results

A3.1. Fertilization

The fertilization results are presented in Fig. 1. A significant difference in fertilization rate between the control (93.89 ± 0.73%) and treatment groups (91.22 ± 0.44%, 91.11 ± 0.67%, 90.44 ± 0.44%, 87.22 ± 0.83%, 78.00 ± 1.26%, respectively) was found ($P < 0.05$). In the treatment groups, the effect of sperm concentration on fertilization was also significant. The highest fertilization rate was achieved with the eggs fertilized by the sperm in the highest pre-freezing concentration (62.5×10^6/ml), and was significantly higher ($P < 0.05$) than the two lowest pre-freezing concentrations (12.5×10^6/ml and 25×10^6/ml).

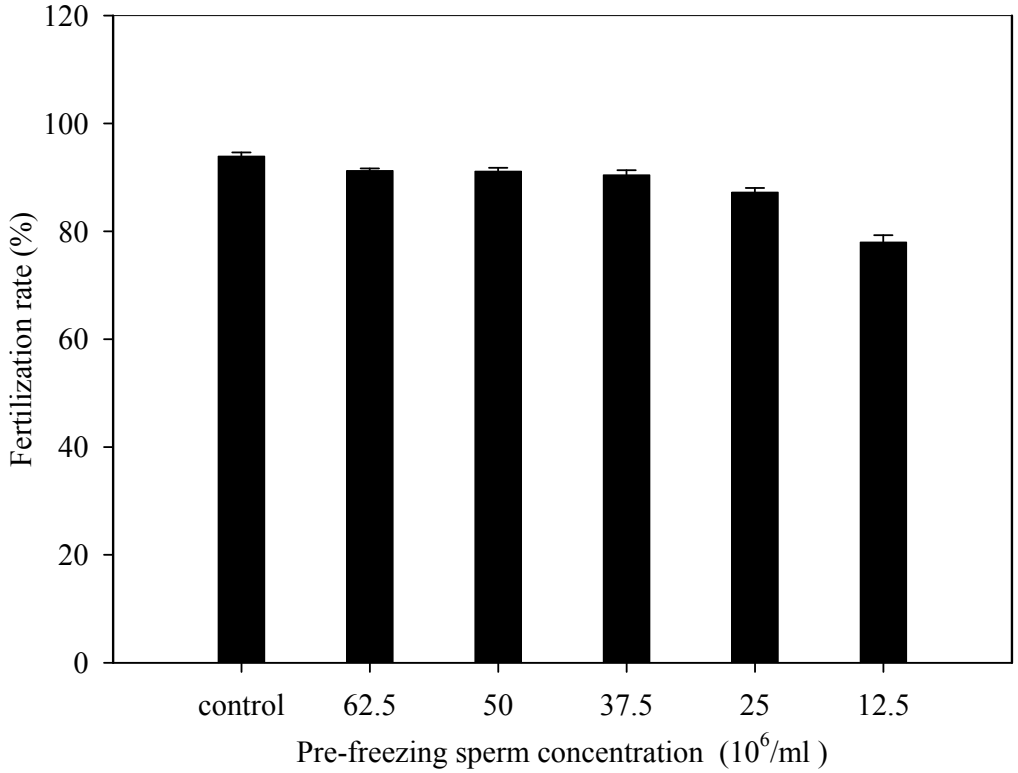

Fig. 1. Fertilization rate obtained with fresh and cryopreserved sperm. The control group was fertilized using fresh sperm

A3.2. Evaluation of sperm quality by flow cytometry

A3.2.1. *Acrosome integrity (FITC-PNA/PI assay)*

On the FTIC-PNA/PI two-dimensional dot plots (Fig. 2), quadrants were set to identify the following three subpopulations: FITC-PNA^+/PI^-green (live sperm with intact acrosome, LR); FITC-PNA^-/PI^+ red (dead sperm, UL) and FITC-PNA^+/PI^+ dual stained (dying sperm with intact acrosome, UR), and no stained particles and cell debris, (LL < 15%) (data not shown).

Cryopreservation induced dramatic changes in acrosome integrity in Pacific oyster sperm (Fig.3). In fresh sperm, the percentage with an intact acrosome (FITC-PNA^+/PI^-) was 87.10 ± 1.57%. This was significantly reduced ($P < 0.05$) to between 20.21 ± 0.63% and 15.80 ± 0.61% in the cryopreserved samples. The number of sperm with FITC-PNA^+/PI^+ significantly increased ($P < 0.05$) from 4.38 ± 0.14% in fresh sperm to 14.03 ± 2.26% to 24.99 ± 1.46% after cryopreservation. Cryopreserved samples also had a higher ($P < 0.05$) percentage (between 51.9 1 ± 3.09% and 53.34 ± 4.28%) of dead sperm (FITC-PNA^-/PI^+) than fresh samples.

In the cryopreserved samples the differences in the percentage of FITC-PNA^+/PI^- and FITC-PNA^+/PI^+ sperm between different groups were significant ($P < 0.05$). The percentage of

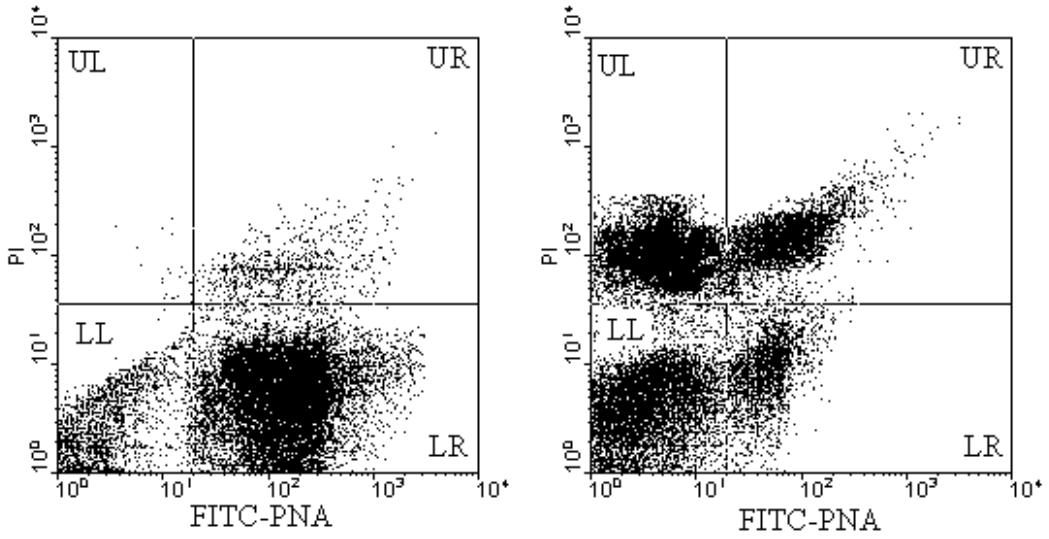

Fig. 2. Flow cytometry dot plots of oyster sperm stained with FITC-PNA and PI
Left: Fresh sperm; Right: cryopreserved sperm

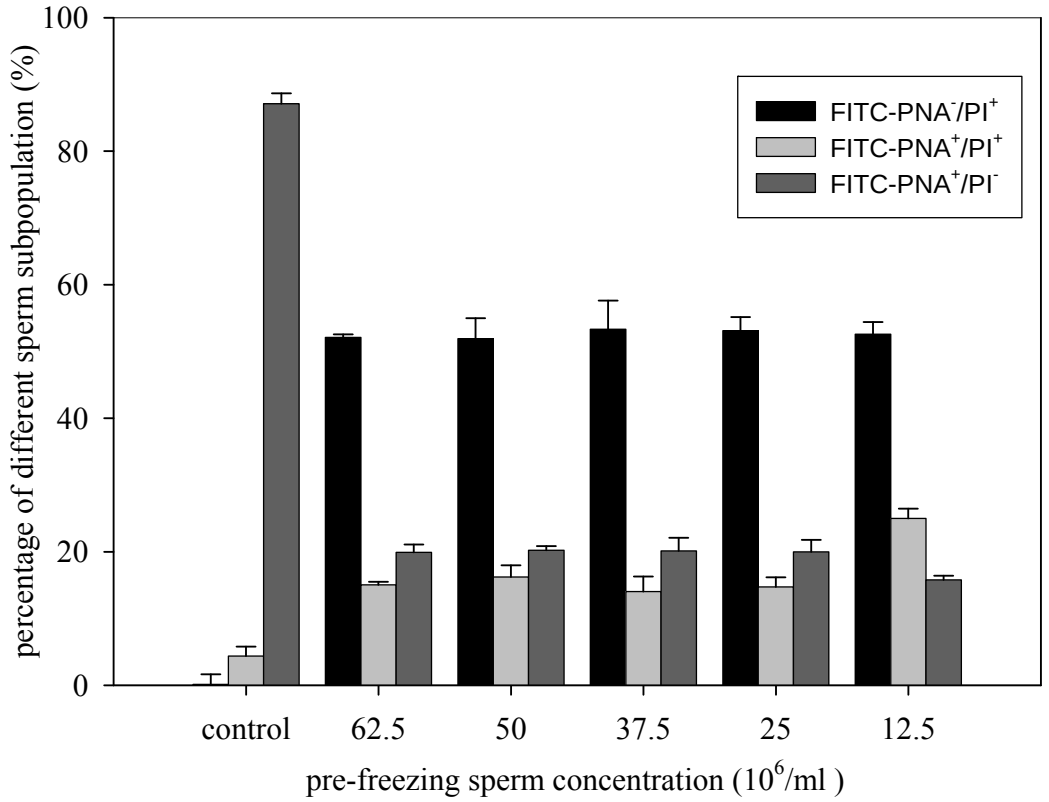

Fig.3. Percentage of oyster sperm subpoplution as evaluated by FITC-PNA and PI staining and flow cytometry. The control group is fresh sperm.

FITC-PNA$^+$/PI$^-$ sperm in the 12.5×10^6/ml group was lower and the percentage of FITC-PNA$^+$/PI$^+$ sperm in the same group much higher than that in the other groups. Interestingly, the percentages of dead sperm with FITC-PNA$^-$/PI$^+$ were evidently not affected (P = 0.953) by the pre-freezing concentration.

3.2.2. Mitochondrial membrane potential (Rh123/PI assay)

Three sperm subpopulations were apparent from the Rh123/PI two-dimensional dot plots (Fig. 4): green, Rh123$^+$/PI$^-$ (live sperm with mitochondria activity, LR); red, Rh123$^-$/PI$^+$ (dead sperm, UL) and dual stained, Rh123$^+$/PI$^+$ (dying sperm with mitochondrial activity, UR). The total from the three sperm subpopulations did not reach 100% because a small group of particles or cell debris (LL) (<10%) did not show any binding (data not shown).

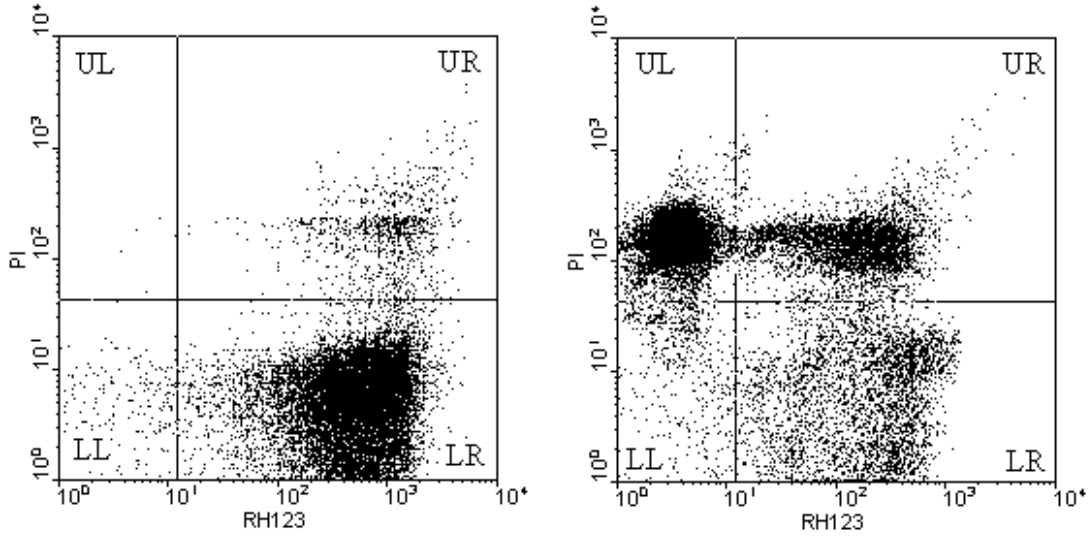

Fig. 4. Flow cytometry dot plots of oyster sperm stained with RH123 and PI.
Left: Fresh sperm; Right: cryopreserved sperm

The mitochondrial membrane potential in cryopreserved sperm significantly decreased ($P < 0.05$) in comparison to fresh sperm (Fig. 5). Fresh sperm samples contained 91.27 ± 1.25% Rh123$^+$/PI$^-$ sperm in total, whereas cryopreserved samples

had between 19.73 ± 1.20% and 34.68 ± 1.98% $Rh123^{+}/PI^{-}$ sperm. In addition, the percentage of $Rh123^{+}/PI^{+}$ and $Rh123^{-}/PI^{+}$ spermatozoa increased significantly ($P < 0.05$) after cryopreservation. The percentage of $Rh123^{+}/PI^{+}$ and $Rh123^{-}/PI^{+}$ spermatozoa increased from 6.34 ± 1.17% and 0.12 ± 0.04% in fresh sperm samples to between 13.97 ± 1.74% and 26.91 ± 1.24% and > 43% in the cryopeserved samples, respectively.

A significant concentration effect ($P < 0.05$) was found between different pre-freezing sperm concentrations in terms of percentages of $Rh123^{+}/PI^{-}$ and $Rh123^{+}/PI^{+}$sperm. The percentage of $Rh123^{+}/PI^{-}$ sperm in the 12.5 × 10^{6}/ml pre-freezing sperm concentration group was significantly lower ($P < 0.05$) and the percentage of $Rh123^{+}/PI^{+}$ sperm in the same group was significantly higher ($P < 0.05$) than in other groups. Surprisingly, the percentages of $Rh123^{-}/PI^{+}$ staining (dead sperm) in cryopreserved samples did not significantly differ ($P = 0.337$) from each other.

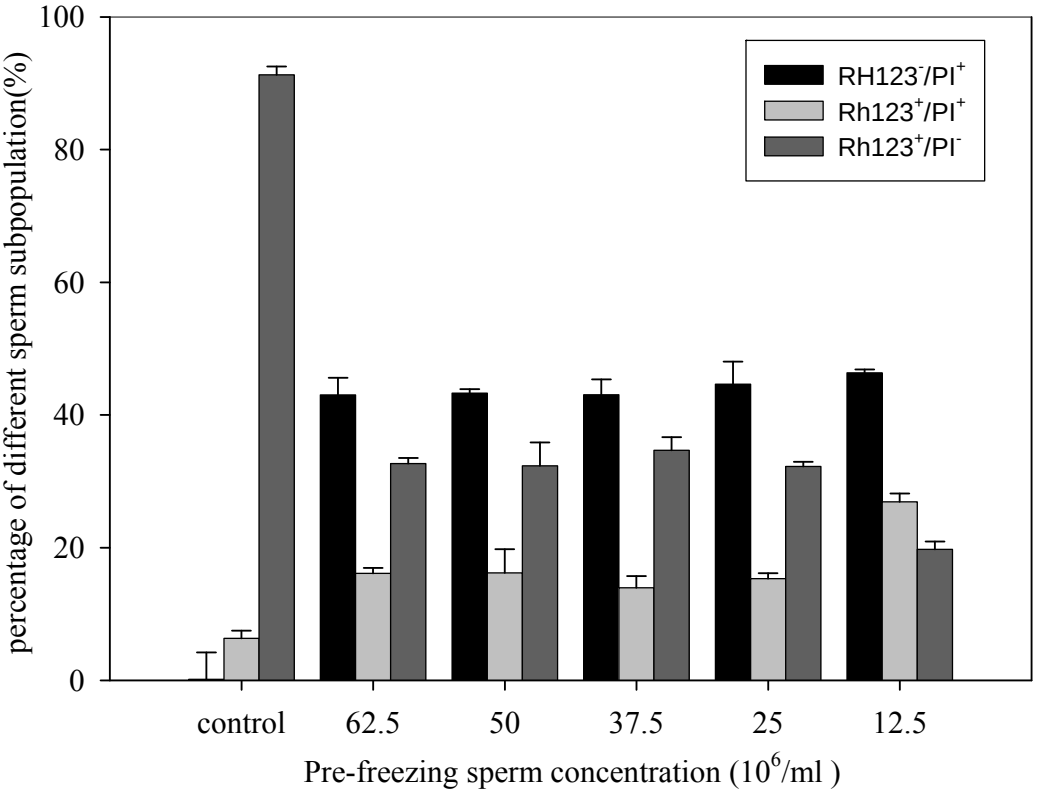

Fig. 5. Percentage of oyster sperm subpoplution as evaluated by Rh123 and PI staining and flow cytometry. The control group is fresh sperm.

A3.3. Correlation between the results from different assays and sperm fertility

Of the oyster sperm quality parameters tested, several were significantly correlated with fertilization rates and the concentration of sperm cryopreserved. Correlations between single tests and fertilization rate and pre-freezing sperm concentrations are summarised in Tables 2and 3. The fertilization rate obtained with cryopreserved sperm was significantly correlated with sperm acrosome parameters indicated by either $Rh123^+/PI^-$ and $Rh123^+/PI^+$ or $Rh123^-/PI^+$ sperm percentages, however, none of the sperm chromatin characteristics were significantly correlated with fertilization rate. For the FITC-PNA/PI assay, only the percentages of FITC-PNA^+/PI^+ and FITC-PNA^+/PI^- sperm significantly correlated with fertilization rate.

Table 2. Correlation coefficients between fertilization rate, pre-freezing sperm concentration and percentages of different sperm subpopulations stained with Rh123 and PI

	Fertilization rate	Pre-freezing concentration	$Rh123^-/PI^+$	$Rh123^+/PI^+$
Pre-freezing concentration	0.858^{**}			
$Rh123^-/PI^+$	-0.555^{*}	-0.514		
$Rh123^+/PI^+$	-0.871^{**}	-0.595^{*}	0.497	
$Rh123^+/PI^-$	0.901^{**}	0.654^{**}	-0.566^{*}	-0.993^{**}

** $P < 0.01$; *$P < 0.05$

Table 3. Correlation coefficients between fertilization rate, pre-freezing sperm concentration and percentages of different sperm subpopulations stained with FITC-PNA and PI

	Fertilization rate	Pre-freezing concentration	PNA^-/PI^+	PNA^+/PI^+
Pre-freezing concentration	0.858^{**}			
PNA^-/PI^+	-0.029	-0.137		
PNA^+/PI^+	-0.884^{**}	-0.611^{*}	-0.204	
PNA^+/PI^-	0.802^{**}	0.587^{*}	-0.287	-0.769^{**}

** $P < 0.01$; *$P < 0.05$

A3.4. Correlation between different parameters

Correlations between different parameters tested in this study were analysed (Table 4). For cryopreserved sperm significant correlations existed between the $Rh123^+/PI^-$ or $Rh123^+/PI^+$ results and FITC-PNA^+/PI^- or FITC-PNA^+/PI^+ results ($r = 0.829$, $P < 0.05$; $r = 0940$, $P < 0.05$, respectively). However, there was no correlation between the $Rh123^-/PI^+$ result and FITC-PNA^-/PI^+ result ($r = 0.411$, $P = 0.128$).

Table 4. Correlation between sperm quality parameters in cryopreserved sperm

	$Rh123^-/PI^+$	$Rh123^+/PI^+$	$Rh123^+/PI^-$	PNA^-/PI^+	PNA^+/PI^+
$Rh123^+/PI^+$	0.497				
$Rh123^+/PI^-$	-0.566*	-0.993**			
PNA^-/PI^+	-0.411	0.076	-0.042		
PNA^+/PI^+	0.635*	0.940**	-0.952**	-0.204	
PNA^+/PI^-	-0.348	-0.830**	0.829**	-0.287	-0.769**

** $P < 0.01$; *$P < 0.05$

A4. Discussion

Cryopreservation usually decreases post-thaw Pacific oyster sperm motility (Kurorura, 1990) and survival to D-larvae (Zhang, 2004). The results of the present study showed that cryopreservation causes extensive damage to Pacific oyster sperm membrane, acrosome, mitochondria and chromatin structure. The results also showed that the correlation between the quality of cryopreserved sperm and fertility was significant.

The Rh123/PI and FITC-PNA/PI assays revealed that the proportion of live sperm decreased significantly after storage in liquid nitrogen, indicating the damaging effects of cryopreservation. In a direct comparison, the percentage of normal acrosome sperm was significantly lower in cryopreserved sperm than in fresh sperm samples. The same was observed for the percentage of sperm with intact mitochondria membranes. This finding is in agreement with similar investigations done in turbot and cattle (Ogier de Baulny, 1997; Ericsson et al., 1993). The low percentage of viable sperm after thawing explained the requirement for a higher sperm/egg ratio when using

cryopreserved sperm compared with fresh sperm in previous experiments by Zhang (2004), since fertilization requires a sufficient number of live sperm with the acrosome functionality and intact mitochondria for the supply of energy. The relationship between sperm quality and fertility is known to be asymptotic and depends on the interaction between the number and the quality of spermatozoa inseminated (Januskauskas et al., 2001). Our results confirmed that the concentration of cryopreserved sperm and percentage of live sperm after cryopreservation significantly correlated to fertility.

Cryopreservation resulted in an increase in the percentage of dead sperm. However, the levels of dead sperm in the 5 pre-freezing sperm concentrations were found to be similar. Interestingly the percentage of dead sperm was just moderately (in Rh123/PI assay) or not (in FITC-PNA/PI assay) correlated with the fertilization capability of the cryopreserved sperm samples. The results also indicated that cryopreservation induced significant increases in the percentage of partly damaged and dying sperm, which were significantly correlated to both fertility and cryopreserved sperm concentration. The percentage of dying sperm in the lowest pre-freezing sperm concentration group (12.5 x 10^6/ml) was higher than that in the pre-freezing groups with higher sperm concentrations. This finding implies that in addition to live and dead sperm a considerable number might exist with altered and possibly dysfunctional plasma membranes. These sperm might also contribute to low fertility, since it is not likely that these cells are functionally competent.

In addition, it was found that the percentage of live sperm were different between treatment groups. The percentage of live sperm in Rh123/PI assay was higher than in the FITC-PNA/PI assay. This result indicates that sperm with mitochondrial defects may be more tolerant to cryopreservation procedures than are those with defects of the acrosome. Different cellular structures seem to have different susceptibility to physicochemical stress. Significant correlations between the Rh123/PI assay and the FITC-PNA/PI assay were also established.

In conclusion, cryopreserving sperm through conventional freezing-thawing procedures resulted in dramatic damages to the sperm plasma membrane, acrosome and mitochondria in Pacific oysters. The results from this study indicated that

outcomes of the Rh123/PI and FITC-PNA/PI assays were significantly correlated with the fertilization rate in cryopreserved sperm samples. These two methods could therefore be applied for routine evaluation of sperm quality and prediction of fertilization success in Pacific oysters.

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