Experimental Production of Tetraploid Oysters for Use as Broodstock for Commercial Hatchery Production of Triploids

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1994/081 Experimental production of tetraploid oysters for use as broodstock for commercial hatchery production of triploids

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OBJECTIVES:

1. The experimental production of tetraploid (4n) oyster embryos, larvae and spat.
2. On-growing of tetraploid (4n) oyster spat to adulthood and reproductive capability.
3. Hybridization of diploid (2n) gametes (from tetraploid, 4n, broodstock) with 1n gametes (from "normal" 2n broodstock) to produce triploid (3n) embryos, larvae and spat.

OUTCOMES ACHIEVED

1. Tetraploid oyster embryos were induced by most methods used in this study; including hydraulic pressure shock, thermal shock, chemical treatment and combined chemical treatment.

2. Tetraploid larvae and spat were produced by the combined chemical method developed in this study.

Non Technical Summary:

Triploids (3n) are organisms with three sets of chromosomes, while the normal diploid (2n) organisms have only two sets. Over the past two decades, triploids have been studied in more than 20 species of molluscs. The main interests of those studies have primarily focused on their sterility and improved growth rates. These are mainly due to that in the normal diploid commercial stocks the animals expend considerable energy on gametogenesis and become watery in the summer months making them less desirable in the market. In Pacific oysters, for example, they release approximately 50% of their body mass during spawning, affecting their meat quality

and acceptance by consumers for extended period of time. In a sterile stock, on the other hand, they could partially redirect this energy to growth and maintain their meat quality during these periods, allowing them to be marketed year round. Triploids, in most species studied so far, experience significantly higher growth rates than their diploid siblings. In bivalve species triploids have showed 10 to 80% faster growth than their diploid siblings (Guo, 1999). However, the expression of the triploid advantage in growth can be influenced by genetic and environmental factors. In addition, production of triploids from normal diploid stock directly is technically difficult in most bivalve species; often resulting in inconsistent percentage of triploid and high larval mortality in the first few days. The commercial use of triploids may, therefore, ultimately depend on the development of tetraploids, which can produce 100 % pure triploids simply by mating with normal diploids.

The establishment of tetraploid breeding stock however, is still a major challenge in most molluscan species. Prior to 1997, tetraploid bivalve spats have only been produced in three species: mussels (17.2% in one month old, Scarpa *et al.* 1993); Manila clam (3 spat detected, Allen *et al.* 1994); and Pacific oysters (67% in juveniles, Guo and Allen 1994). The tetraploid mussels and clams were induced using gametes from diploid males and females and the tetraploid Pacific oysters were produced using eggs from triploid females and sperm from diploid males.

The main objectives of this project were to evaluate and develop techniques for the production of tetraploid broodstock in Pacific oysters and to investigate the potential to produce triploids by crossing tetraploids with diploids. Throughout this project (March 1995 to February 1998), most techniques developed to induce tetraploids from diploid stock in fish and shellfish, have been attempted; new techniques were investigated; and tetraploid spats were produced. The techniques published by Guo and Allen (1994) was not tried because no mature triploid broodstock were available in South Australia.

The first objective "the experimental production of tetraploid (4n) oyster embryos, larvae and spat" was achieved. Tetraploid embryos were induced by most methods used in this study; including, 1) electrofusion of cells in two cell stage embryos; 2) thermal, and heat + caffeine treatments, to inhibit first mitotic division; 3) hydraulic pressure treatments to prevent first mitotic division; 4) Cytochalasin B (CB) inhibition of first polar body formation or both polar body formations; 5) CB and 6-dimethylaminopurine (6-DMAP) inhibition of first mitotic division; and 6) combined chemicals treatment. The majority of tetraploid embryos produced in this study (29 % or more) were produced by the hydraulic pressure treatment and the combined chemical methods. Flow cytometric analysis was used to identify tetraploid spat. Analysis from experiments in the second (23 February 1996) and third (25 February 1997) year of the project

identified 1 tetraploid in 28 and 79 spat tested respectively. In general, tetraploid levels in embryos were initially high, however, these were not stable; as embryos developed, the ploidy levels decreased.

The second objective "on-growing of tetraploid oyster spats to adulthood and reproductive capability" was partially achieved. In the third year of the project, March 1997, approximately 2 million, eight-day-old larvae (8 % tetraploids) and 150 twelve month old spats (4% tetraploids, as analysed by flow cytometry; n = 28), were sent to the South Australian Oyster Hatchery for grow-out. A sub-sample from the larval batch was also reared at Flinders University. The spat (12 months old) from this batch were sampled towards the end of the third year of the project (January 1998) using flow cytometric analysis. Non-destructive ploidy assessment of spat is not possible and therefore precise estimates of the proportion of tetraploids in the spat being grown out on oyster farms are not available. The percentage of tetraploids growing out on farms can only be estimated based on the samples analysed at Flinders University. At the time of writing the initial report draft, August 1999, the reproductive capability of oysters grown out at the South Australian Oyster Hatchery had not been obtained, however they are being maintained and checked periodically for indications of sexual maturity.

During the second year of the project (July 1997), the industry was consulted about the direction of oyster research at the South Australian Oyster Growers Association (SAOGA) Field Day, Smoky Bay. At this time the industry indicated that grower demand for triploid oysters had fallen considerably in the light of poor growth rates and meat quality of chemically produced triploid oysters on commercial leases. As a result, plans for continued research into production of tetraploid broodstock were abandoned.

The last objective "hybridization of diploid gametes (from tetraploid broodstock) with 1n gametes (from "normal" diploid broodstock) to produce triploid embryos, larvae and spats," was not achieved due to non-completion of the second objective.

Unfortunately, the ploidy levels and the performance of the stocks sent to the South Australian Oyster Hatchery for grow-out were not collected. If enough tetraploids were produced and both male and female existed in the tetraploid stock, the breeding tetraploid line could be established by mating between them. In United States the second generation Pacific oyster tetraploids have already been established by mating the tetraploids produced using the eggs from triploid females (Guo *et al.* 1996).

Although very low percentages of tetraploid Pacific oyster spats were induced in some experiments, the success of this study also indicates that the development of zygotes, produced by fertilising eggs from diploid females with sperm from diploid males, could tolerate tetraploid genome in their cells.

KEYWORDS: Pacific oyster, *Crassostrea gigas*, ploidy manipulation, tetraploid.

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BACKGROUND

The sale of commercially cultured oysters grown in Australia generated approximately $53.4 million in 1996/97. These oysters, primarily the Sydney rock oyster *Saccostrea commercialis* and the Pacific oyster *Crassostrea gigas*, have traditionally been cultivated from larvae and spat generated from "normal diploid" (*i.e.* genetically unmanipulated) broodstock.

Within the last two decades there has a considerable amount of research on the production of genetically manipulated stocks, especially triploids. Triploids are individuals that have three complete sets of chromosomes (3n) rather than the two sets found in most normal diploid (2n) organisms. The advantage of producing triploids stems from their inability to develop gonads; triploid individuals divert almost all of their energy to growth and are correspondingly larger than reproductively active diploid stocks of a similar age. The commercial benefits of generating triploid stocks are thus considerable and some aquacultural organisations are now reaping the rewards of this technology (Guo, 1999).

Triploidy-induction techniques have recently been applied to the shellfish industry, particularly to the culture of oysters (Guo *et al.* 1994). Oysters are almost completely unsaleable during their reproductive season, which can extend for 4 – 6 months of the year. Therefore the potential benefits of producing triploid individuals are considerable.

The induction of triploidy in Pacific oysters has become the subject of much aquacultural research, and reliable high-yield techniques are currently being developed (*e.g.* Allen and Downing 1990; Guo *et al.* 1994; Beaumont and Fairbrother 1991). Most of this research has focussed on the production of triploids by manipulating the normal process of egg maturation and development. The methods used apply sub-lethal concentrations of (toxic) chemicals into newly fertilised oyster eggs, to inhibit meiotic division. These very small quantities of cytotoxin prevent normal maturation of eggs by inhibiting polar body formation, resulting in triploid instead of diploid embryos. This process, combined with optimal environmental conditions, has produced reliable yields in excess of 90 % triploids per batch. However these induced triploids sometimes have relatively high mortality rates, causing the proportion of "normal" diploids in a batch ready for harvest, to increase up to 10-20 %. This poses a problem for commercial farmers who bring triploid batches to market during the lucrative Christmas season, when fresh diploid oysters are usually unavailable due to their reproductive condition. The incidence of consumers purchasing unpalatable diploids can severely erode market confidence in the product.

An additional difficulty with chemically manipulated triploid oysters is that although very small quantities of chemicals are used, those chemicals are highly toxic. From a practical standpoint,

the subsequent grow-out of triploid oysters in toxin-free environments removes all traces of those chemicals from the oysters. Recent work in Canada, France and Australia also indicates that cheaper and less toxic chemical treatments may be available (Nell *et al.* 1996; Desrosiers *et al.* 1993; Gerard *et al.* 1994). However, it remains clear that chemically generated triploid oysters may nonetheless encounter some degree of consumer resistance in the marketplace.

To date a small number of viable tetraploid spats have been obtained by manipulating meiosis in eggs from four diploid bivalve species, including, blue mussel (Scarpa *et al.* 1993); Manila clam (Allen *et al.* 1994); Zhikong scallop (Yang *et al.* 2000) and dwarf surfclam (Peruzzi and Guo 2002). Studies by Allen *et al.* (1994) and Komaru *et al.* (1995) also sampled one-year-old mature male mussels and Manila clams; analysis showed that gametes contained approximately twice the amount of DNA compared to sperm from diploids. However, the successes documented so far have been difficult to reproduce and no breeding populations of tetraploids have been established from them (Peruzzi and Guo 2002). The same Zhikong scallop population was also re-sampled at a later date and no tetraploids were identified (Yang *et al.* 2000). The inability to identify tetraploids at a later date may be due to the reversal of tetraploids to mosaics or lower ploidy levels. In the eastern oyster, *Crassostrea virginica,* at five months post fertilisation about 10 % of the tetraploids (produced by inhibiting the formation of the first polar body in eggs from triploids fertilised with normal sperm) changed to primarily triploid/tetraploid mosaics (Guo *et al.* 2002). Reductions in triploid levels of ≥20 % have also been reported in Pacific oysters (Allen *et al.* 1996).

A different method of tetraploid induction was developed in the Pacific oyster in 1993, which produced 2000 viable tetraploids, by inhibiting the formation of the first polar body in eggs from triploid females. This method also produced small numbers of tetraploids in the pearl oyster *Pinctada martensi* (He *et al.* 2000) and large numbers of tetraploid spat in the Pacific and eastern oysters (Eudeline *et al.* 2000; Guo *et al.* 2002). Now, triploid Pacific oysters, produced by sperm from tetraploid broodstock, are commercially farmed on the West Coast of North America, France (Nell 2002).

This project was conducted between March 1995 and February 1998. However, due to project staff changes preparation of the final report was delayed.

NEED

The commercial application of current methods for producing triploid oysters is hampered by sub-optimal triploid yields and the possibility of public apprehension over chemically manipulated foodstuffs. The ability to produce 100 % triploid oyster spat without chemical manipulation would result in substantial increases in the sales of Australian cultivated oysters.

The research reported here aimed to develop methodologies to produce tetraploid (4n) oyster broodstock. Those broodstock were then to be crossed with "normal" diploid (2n) broodstock in a hatchery to produce triploid (3n) embryos and larvae. This approach removes the stigma of chemical manipulation from product, whilst simultaneously ensuring 100 % triploid yield.

OBJECTIVES

1. The experimental production of tetraploid oyster embryos, larvae and spat.
2. On-growing of tetraploid oyster spats to adulthood and reproductive capability.
3. Hybridization of diploid gametes (from tetraploid broodstock) with 1n gametes (from "normal" diploid broodstock) to produce triploid embryos, larvae and spats.

MATERIALS AND METHODS

Method 1 – Electrofusion

Electrofusion, the technique used in these experiments to fuse oyster cells, relies on electrical disruption of membranes of cells that are in close contact. The application of pulsed strong electric fields across the longitudinal axis of a 2-cell embryo causes variable poration of the membrane, leading to loss of integrity of the dividing cell wall and fusion of the two cells (Cadoret 1992). Subsequent fusion of the two diploid nuclei creates a tetraploid nucleus. This method is highly sensitive to the orientation of the embryo with respect to the electrodes.

Electrofusion device

A high-voltage pulse generator (Grass Stimulator) was used to deliver rectangular (square-wave) pulses. The voltage, field pulse duration and pulse shape were controlled by an electronic oscilloscope. The field strength was then calculated from the voltage applied and the distance between the two electrodes.

The fusion chamber was designed to be observed through an optical microscope. Two parallel platinum wires located about 1 mm apart served as electrodes, were glued at both ends to a microscope glass slide. These electrodes, and their support glass, formed the bed of an open chamber (Fig. 1).

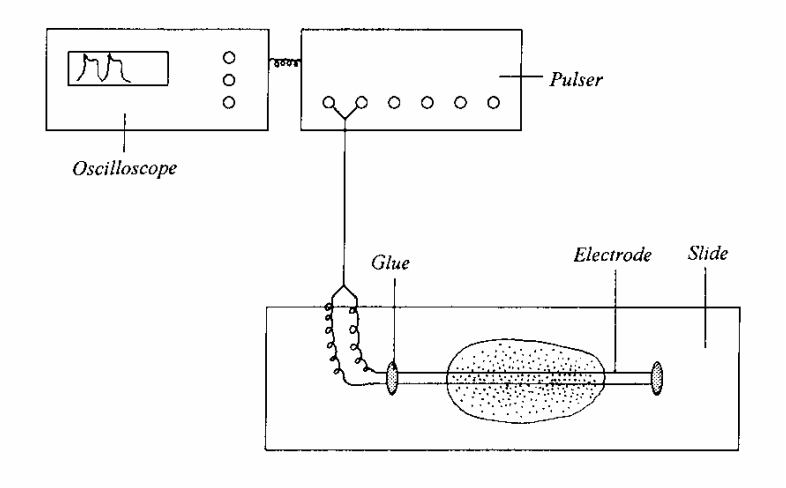

Fig. 1. Electrofusion device for fusing oyster embryos.

Electrofusion medium

The electrofusion medium used was a solution of 0.6 M sucrose in distilled water. Sucrose concentrations higher than 0.6 M were not used as they were more dense than the embryos (Cadoret 1992), which either caused embryos to float or take too long to settle in the chamber. The survival and normal development of the oyster oocytes or early embryos kept in several sucrose concentrations (0.6 to 1.0 mM), for different durations (5 to 30 min), showed differences to those in seawater controls (Cadoret 1992).

Gamete collection

Broodstock were supplied by oyster farmers at Smoky Bay or bought from the local fish markets in Adelaide (South Australian oysters). Oysters were kept in individual beakers and spawned by thermal stimulation or strip-spawned (Li and Havenhand unpubl.).

Tetraploid inductions

The experiments were conducted in late 1995.

Gametes from at least two females were fertilized within 2 h post-spawning with sperm from two to three males, at an egg/sperm ratio of about 1 egg/15 sperm (assayed by direct observation under the microscope). Eggs and sperm were pooled separately before fertilisation. After 10 min, fertilized eggs were washed with filtered seawater to remove the excess sperm and kept in 0.22 μm filtered seawater at 25 ^{o}C.

Earlier trials demonstrated that effective electrofusion was obtained at a field strength of 600 Vcm^{-1} with a duration of 50 μs applied for 2 times. Approximately two hundred, 2-cell stage embryos were placed randomly between the electrodes in the electrofusion medium. Five minutes later, approximately 75 - 80 min post-fertilisation, electrical pulses were applied. The courses of fusion and re-division of the fused embryos was monitored closely under the microscope.

Method 2 – Thermal Shock and Thermal + Caffeine Shock

Thermal shock

Both heat and cold shocks can be used to interrupt mitotic spindle formation at first cleavage to produce tetraploids and could thus provide an easy and safe method for induction of tetraploids.

However, since thermal treatment arrests all development, only those eggs that are at a vulnerable stage of cell division at the time of shock, are affected by the treatment in this way (Allen 1987).

Thermal + caffeine shock

A combined thermal + caffeine shock technique to induce triploidy in bivalves was developed by Yamamoto *et al.* (1990) and evaluated by Scarpa *et al.* (1994). Observations in Pacific oysters and blue mussels using this combined technique have shown marked increases in triploidy inductions and relatively good survival rates, in comparison to using the two agents separately (Yamamoto *et al.* 1990).

Gamete collection

Broodstock were provided by the South Australian Oyster Hatchery at Port Lincoln, where the oysters were conditioned for commercial-scale spat production. Gametes were obtained by dissecting gonads of matured animals. Eggs were passed through a 70 μm screen to remove tissue debris and rinsed on a 15 μm screen. A 37 μm screen was used in filtering sperm suspension.

Tetraploid inductions

The experiments were conducted in August and September 1996 and December 1997.

Eggs were fertilized at 25 ^{o}C according to the methods outlined above (Method 1: Tetraploid inductions). Thermal treatments consisted of four temperatures: 2 ^{o}C; 32 ^{o}C; 34 ^{o}C and 35 ^{o}C. The combined heat + caffeine treatment consisted of 15 mM caffeine at 32 ^{o}C (Yamamoto *et al.* 1990; Scarpa *et al.* 1994). These treatments were applied for a period of 15 min, once the majority of fertilised zygotes had expelled polar body II. This usually occurred about 55 min post-fertilization at 25 ^{o}C. Temperatures for thermal treatments were selected based on the results from previous studies investigating ploidy manipulations in molluscs and other animals (Guo *et al.* 1994; Quillet and Paneley 1986; Arai *et al.* 1986 and Thorgaard *et al.* 1981). In total there were five treatments, with 3 replicates and a total of five hundred thousand eggs per treatment. Replicates within each treatment were from the same batch of pooled eggs. Following treatments, embryos were kept in filtered seawater at a density of 50 embryos/mL.

Method 3 – Pressure Shock

This method was not included in the original proposal, however hydrostatic pressure shocks have been successfully used in producing tetraploid fish (Chourrout 1984). Pressure shocks have

similar limitations to thermal shocks; *i.e.* the effective induction window is very narrow (Beaumont and Fairbrother 1991). As in thermal treatments, development resumes when the shock is released.

Gamete collection

Broodstock supplied by South Australian oyster growers were bought from the Adelaide fish markets and held in recirculating seawater systems at Flinders University. Eggs and sperm were pipetted from the gonads of the matured animals. Eggs in suspension were filtered with two screens (70 μm screen on top; 15 μm on bottom) to remove the excess gonadal debris. Two females and two males were used in each experiment.

Tetraploid inductions

Tetraploid inductions using pressure shock treatments were conducted in spring and summer of 1996/97 and 1997.

Pressure shock inductions were obtained with the aid of a custom-built 200 mL stainless steel pressurising chamber. Eggs were fertilised according to the methods outlined in Method 1: Electrofusion, then held in a 2 L beaker at 25 ^{o}C. Approximately 100,000 zygotes suspended in seawater were pipetted into two, 25 mL containers (50,000/container) and sealed with Parafilm. One container was placed into the pressure chamber for pressure treatment; the other was used as control. Pressures of 300±20 kg/cm^2, 350±20 kg/cm^2, 400±20 kg/cm^2, 450±20 kg/cm^2, 500±20 kg/cm^2, 550±20 kg/cm^2 and 600±20 kg/cm^2 were applied at 45, 50, 55, 60 and 65 min post-fertilisation. All pressure treatments were applied for a period of 10 min. These protocols were based on previous experiments on the same species that used different equipment (Li, unpubl.).

The pressure equipment at Flinders University could not stabilise the high pressures for the duration required in these experiments. To overcome this, repeated additional pressure had to be added in order to maintain the required pressure(s). Thus, the pressure intensities used in this study consisted of two parts: an initial pressure and an extra pressure.

Method 4 – Chemical Shocks

Gamete collection

Broodstock were spawned by thermal stimulation or strip-spawning. Strip-spawned eggs were suspended in seawater and filtered with two screens (70 μm screen on top; 15 μm on bottom) to

remove excess gonadal debris. Two females and two males were used in small-scale experiments, while at least four females and three males were used in large-scale experiments. At least 50,000 or 2,000,000 eggs were treated in small-scale and large-scale experiments respectively.

Tetraploid inductions

A temperature of 25±1 °C was maintained consistently throughout the treatments. Four methods were tested. In all experiments, a replicate from one batch was treated only with 0.05 % dimethylsulfoxide (DMSO) and kept as a control.

Method 4.1 – Suppression of both polar body formations

(October, 1995, March, 1996 and January, 1997)

The method used by Scarpa *et al.* (1993) to induce tetraploid mussels, was attempted in this project. Both polar body I and II were suppressed by adding CB to the seawater at 7 min post-fertilisation for a period of 35 or 38 min. CB concentrations of 0.5 mg and 1 mg CB/L in 0.05 % DMSO were used in different treatments respectively. After treatment eggs were rinsed in 0.05 % DMSO for another 25 min, washed with filtered seawater and then stocked in 2 L beakers or 18 L plastic containers at a density of 25 ~ 50 embryos/mL.

Method 4.2 – Suppression of polar body I formation

(January and February, 1997)

The method developed by Stephens and Downing (1988) was used in January and February 1997. Eggs pooled from three females were treated with 0.5 mg/L CB in 0.05 % DMSO from 5 min post-fertilisation at 25 °C for 15 min or from 15 min post-fertilisation at 18 °C for 20 min. Eggs were then rinsed in 0.05 % DMSO for at least 20 min and stocked in 2 L beakers at a density of 50 embryos/mL.

Method 4.3 – Suppression of the first mitotic division

(February, November and December 1996)

The induction of tetraploidy was trialled by suppressing the first mitotic division with both CB and 6-dimethylaminopurine (6-DMAP). The 6-DMAP concentration of 450 μmol/L used in this study was suggested by Gerard et al. (1994) as the best dose to induce triploidy in Pacific oysters.

Pooled eggs from three females were fertilized with sperm from two males. Eggs were washed with seawater after a period of 10 min to remove the excess sperm and stirred to keep them in suspension. For tetraploid inductions with 6-DMAP, oyster embryos were incubated in 450 μmol/L 6-DMAP from 45 min post-fertilisation for 25 min at 25 °C. The embryos were then washed thoroughly with filtered seawater. For CB tetraploid inductions the embryos were treated with 0.5 mg/L CB in 0.05 % DMSO from 50 min post-fertilisation for 20 min at 25 °C and then rinsed in 0.05 % DMSO for 20 min. Following the treatments with 6-DMAP or CB, the embryos were reared at a density of about 50 embryos (or larvae)/mL in 18 L plastic tanks.

Method 4.4 – Tetraploidy inductions by combined chemicals

(1995, 1996 and 1997)

This method was not included in the original proposal; it was developed by the project investigators as part of this study.

A series of experiments were carried out from July 1995 to November 1997 to establish this method and to optimise the parameters used in tetraploid inductions. Both blue mussels (*Mytilus edulis,* only used in 1995) and oysters had were used. Eggs from at least two females were fertilised with sperm from two to five males. Eggs were incubated in mixed chemicals (CB and 2-mercaptoethanol), washed and then stocked at a density of 50~100 embryos (larvae)/mL. For details refer to the accompanying confidential document.

General

Prior to spawning, the oysters were sexed and egg quality was checked by gonad biopsy. No hermaphroditic individuals were found in the broodstock used for the experiments.

Larval rearing

Eggs/larvae were incubated at high densities (>50/mL); after a period of 20 h, D-shaped veligers were transferred to 18L spring water containers or in upweller systems modified from those

containers at a density of 10-15/mL. The density was reduced to 5-8/mL at five days post-fertilisation and further reduced to 5/mL at ten days post-fertilisation. Seawater for all tetraploid inductions and larval rearing was filtered to 1 μm. When required an antibiotic, chloramphenicol at a final concentration of 0.5μg/mL (prepared as a high concentration solution and stored in the freezer), was used to control bacterial contamination. Seawater was maintained at 25±1 °C. A complete water exchange occurred daily. A mesh screen was used to retain larvae. 2 h after a water exchange, a mixture of algal species (*Isochrysis galbana, Pavlova lutheri* and *Tetraselmis suecica*) was added to tanks at approximately 20,000 cells/mL (only *I. galbana* and *P. lutheri* at this stage) up to day five; 40,000 cells/mL up to day ten; and 60,000 cells/mL until the pediveliger stage.

Following treatment with combined chemicals in February 1997, ploidy levels were verified using flow cytometry (see below) on day 1 and day 5 respectively, and a batch of larvae (approximately 2 million) were sent to the South Australian Oyster Hatchery, Port Lincoln. Larvae were reared at the commercial hatchery using standard methods.

Metamorphosis

The method developed by Coon *et al.* (1986) to produce cultchless oyster spat was used in this study. In brief, from day 16, larvae larger than 250μm were screened out every other day and treated with 10^{-4} M epinephrine for about 10 h. Epinephrine stock solutions of 10 time higher than the concentration used at the treatment were prepared in 0.005 N HCl in fresh filtered seawater immediately before use. The larvae still swimming after treatment was re-treated two days later with the next group from the same batch. The cultchless spat produced were kept in either a static system for a few days, then stocked into upweller systems; or stocked in the upweller systems directly. The spat were fed twice a day with an algal mixture of *Isochrysis galbana, Pavlova lutheri* and *Tetraselmis suecica*. Three months later, the spat were transferred to a recirculating aquarium system at Flinders University and fed on an algal mixture (see larval rearing) every other day.

Ploidy verification

The ploidy status of control and treated trochophore larvae was determined by either direct chromosome count or flow cytometry. Ploidy status beyond the trochophore stage was determined by flow cytometry only.

1. Direct chromosome count

Techniques for chromosome preparations were modified from Allen *et al.* (1989). At approximately 5 h post-fertilisation, embryo samples were treated with 0.01 % colchicine in filtered seawater for 3 h. They were then exposed to 0.6 % sodium citrate solution, a hypotonic solution, for 20 min. To prevent cells bursting when exposed directly to the Carnoy's the embryos were pre-treated with Carnoy's fixative and 0.7 % sodium citrate solution for a few minutes at a ratio of 1 part of Carnoy's and 9 parts of sodium citrate solution. The embryos were then fixed in full strength Carnoy's (3:1 absolute methanol: glacial acetic acid) for 1 h. During that period, the fixative solution was changed three times. Three to five samples of cells in suspension from each treatment were dropped onto warm glass slides and left to dry at ambient temperature. These slides were then stained in 10 % Giemsa solution for 20 min. The chromosome numbers of at least 40 cells with well spread metaphase were counted under a microscope. Each embryo was only counted once. The Pacific oyster has a diploid chromosome number of 20 (Ahmed 1973). In the present study, the ploidies were classified according to the following: haploid, 9-10 chromosomes; diploid, 18-20; triploid, 28-30; tetraploid, 35-40; and pentaploid, 45-50. Other chromosome numbers were considered as aneuploids.

2. Flow cytometry

Direct chromosome counting is the most reliable method in ploidy verification and results from this method can be used as a standard to compare other ploidy verification methods against; however, this method is time consuming, and applicable only to trochophore larvae and individuals large enough for biopsy assessment. In contrast, flow cytometry analysis is simple, fast, and can be applied to any developmental stage. Therefore, flow cytometry analysis was adopted as the main method for ploidy determination in this study.

For stages before metamorphosis, approximately 2,000~10,000 larvae were collected on a mesh, placed at the centre of a Petri dish and gently crushed with the edge of a standard microscope slide. The slide was then rinsed into a dish with 2 mL phosphate buffered saline (PBS). A similar procedure was applied to the spat, whereby, the top shell of an oyster was pried off and either all or part of the gill was removed; alternatively in small spat, the entire tissue mass was used. The tissue was minced with a scalpel in one to two drops of PBS. The minced sample of spat or larvae was drawn into a 10 mL centrifuge tube and centrifuged at 3,000 rpm for 5 min. The supernatant was decanted and the remaining pellet was resuspended in approximately 1.5 mL of propidium iodide (PI) staining solution for half an hour and screened through a 20 μm mesh to

remove large cell aggregates before being analysed. The PI solution was prepared by mixing 500 mg propidium iodide into 100 mL PBS with 1 % Triton X-100 (SigmaUltra).

Samples were run on a FACScan flow cytometry instrument (Becton Dickinson, California), at Flinders Medical Centre. The argon gas laser was tuned to 488nm. For each sample, the fluorescence of the stained nucleus was determined for each of 20,000 cells. Each cell was categorised according to the amount of fluorescence and allocated to one of 255 channels. At the end of each run, a frequency curve was generated by plotting the number of cells in each channel against the intensity of DNA fluorescence (Fig. 2). The ploidy status of each fluorescence peak in the sample prepared from treated larvae (or a spat) was determined by comparing it to the main peak (2n) in the control sample (Fig. 2A). Fig. 2 shows the flow cytometry results from the larval samples in a tetraploid induction experiment. The mean intensity of fluorescence of the main peak in the control (2n) is 70.65 (Fig. 2A). The mean intensity of fluorescence of the first peak in the treated group is 71.14, which is similar to the mean intensity of fluorescence in the control and is therefore representing diploid larval cells (Fig. 2B). The second peak is 103.97, which is 1.5 times the mean intensity of fluorescence in the control and represents triploid larval cells. The third peak is 134.48, about 2 times the mean intensity of fluorescence in the control, and represents tetraploid larval cells.

In this study the success of each tetraploid treatment was accessed according to the percentage of tetraploid produced at 14 h post fertilisation. This percentage can be calculated by dividing the events in the tetraploid peak with the total events counted (referred as ungated analysis in this study), or with the events within the channel ranges defined (gated analysis). For example, the tetraploid percentage in Fig. 2B is 23.64% with ungated analysis [4769 (range 3) ÷ 20000 (range 0) x 100] or 32.54% [4769 (range 3) ÷14654 (range 4) x 100] when the channel ranges are defined between 55 and 155.

The ploidy level of individual spat is determined by comparing its mean intensity of DNA fluorescence with that of the control.

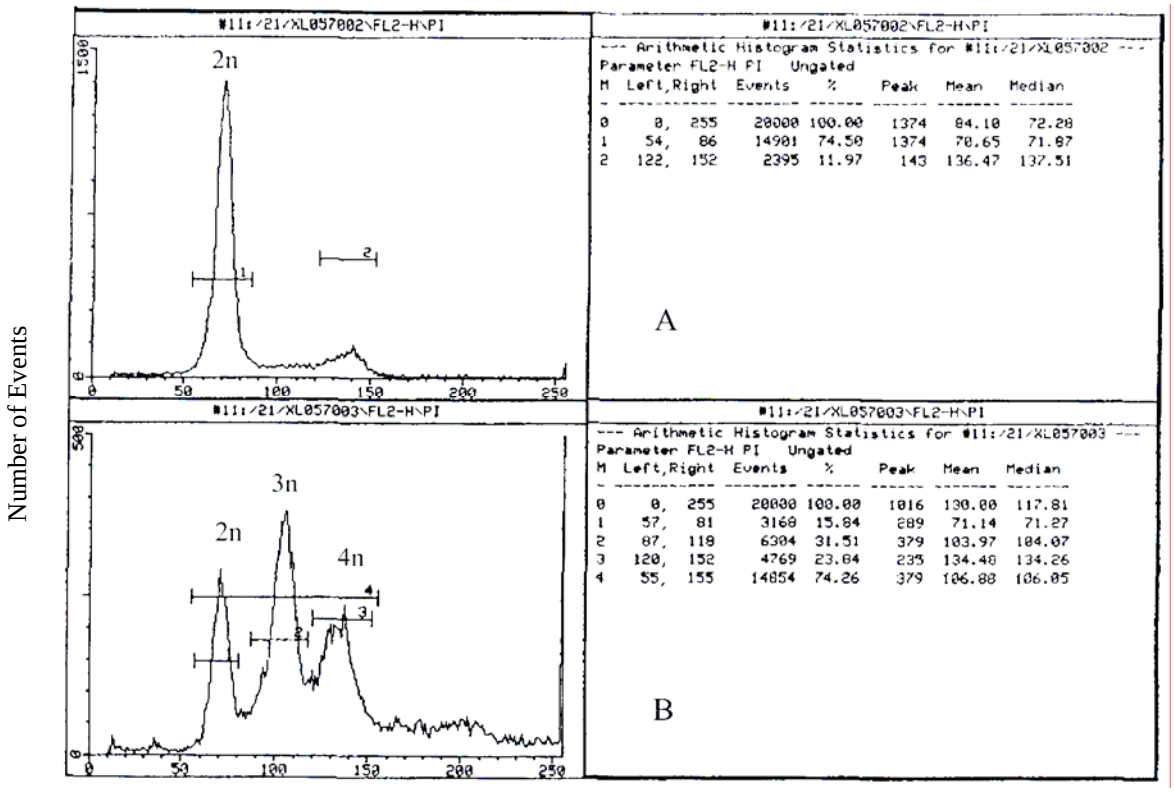

Fig. 2. Frequency curves of the cells relative to their DNA fluorescence from Pacific oyster larvae. A: control; B: chemical treated larvae. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

RESULTS

The normal course of events between egg maturation and the first mitotic division in Pacific oysters is shown diagrammatically in Fig. 3A. After fertilisation the first maturation division (meiotic I division) starts, resulting in a polar body (Pb I) that contains two sets of maternal chromosomes. The second maturation division (meiotic II division) follows, resulting in another polar body (Pb II) containing one set of maternal chromosomes. Only one set of maternal chromosomes (haploid) remains in the nucleus of the egg. When the haploid sperm pronucleus fuses with the haploid egg nucleus a diploid zygote is formed.

Tetraploids can, theoretically, be induced by physical or chemical shock treatment at meiotic I division, first mitotic division or early in the two cell embryo stage. If chemical treatment is administered during the meiotic I division, the maternal chromosomes that should be released in the first polar body are retained within the nucleus of the egg (Fig. 3B). During the meiotic II division, if one set of maternal chromosomes is released with the second polar body, the egg nucleus should contain the other three maternal sets (triploid). When the haploid sperm pronucleus fuses with the triploid egg nucleus a tetraploid zygote is formed (Fig. 3B). The progeny developed from this zygote would be tetraploid. However, if two, three or incomplete sets of chromosomes are released with the second polar body, the resultant zygote should be a triploid, diploid or aneuploid, respectively.

If physical or chemical shock treatment is administered during the 1st mitotic division (Fig. 3C) the chromosomes will divide to form two sets but the cell will not cleave, resulting in one cell with four sets of chromosomes (tetraploid).

If physical or chemical shock treatment is administered between the 1st and 2nd mitotic divisions the two diploid blastomeres can be fused, resulting in a tetraploid cell (Fig. 4c).

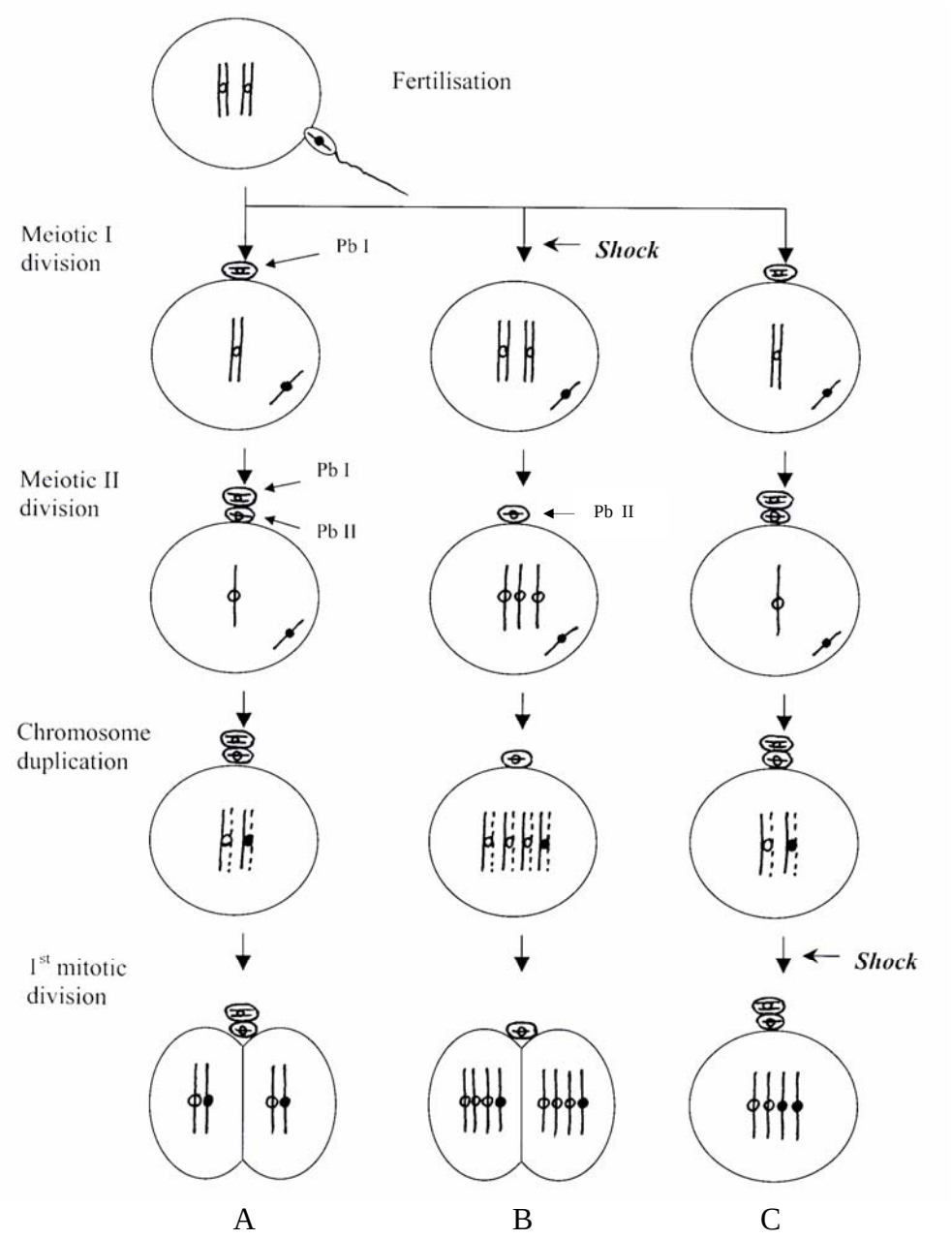

Fig. 3. Diagram of normal egg development and tetraploidy manipulation in Pacific oysters. For simplicity only one pair of chromosomes is shown. A: normal development (diploid); B: inhibiting meiotic I division; C: inhibiting 1st mitotic division.

In this study, only egg batches with a high fertilisation rate (>98%) were used; experiment(s) with egg batches with a fertilisation rate <98 % were abandoned. In control groups, no triploids, tetraploids or aneuploids were found by using the direct chromosome count method. Exposure to both chemical and physical treatments slowed the development of fertilised eggs, causing abnormal development in some larvae, and generally resulted in higher mortality in treated groups than in the controls. Except in combined chemical treatments, no evident tetraploids could be detected with flow cytometry at later larval stages in all other treatments. Individual

tetraploid spat were only confirmed by flow cytometry analysis in the small size grades of the two combined chemical treatment experiments.

Electrofusion

After incubation in filtered seawater, the 2-cell stage embryos of the Pacific oysters were exposed to the electrical pulses in a 0.6 M sucrose solution. A 600 Vcm^{-1} field strength for a duration of 2 X 50 μs, was used. All embryos tolerated the treatments, however only those 2-cell stage embryos with their mutual longitudinal axis perpendicular to the electrodes, were fused (Fig. 4). The signs of fusion were seen as early as 2 min after applying the pulse. The boundaries between the two blastomeres became blunt and then disappeared. At this stage, the movements of the cytoplasm between those two cells, was visible. The resulting 1-cell embryos were oval in shape at first and then rounded out completely. In each trial, slide areas with more than 5 embryos that oriented perpendicularly to the electrodes were focused and observed under the microscope. In six experiments, fourteen embryos clearly fused; four of them continued to redivide.

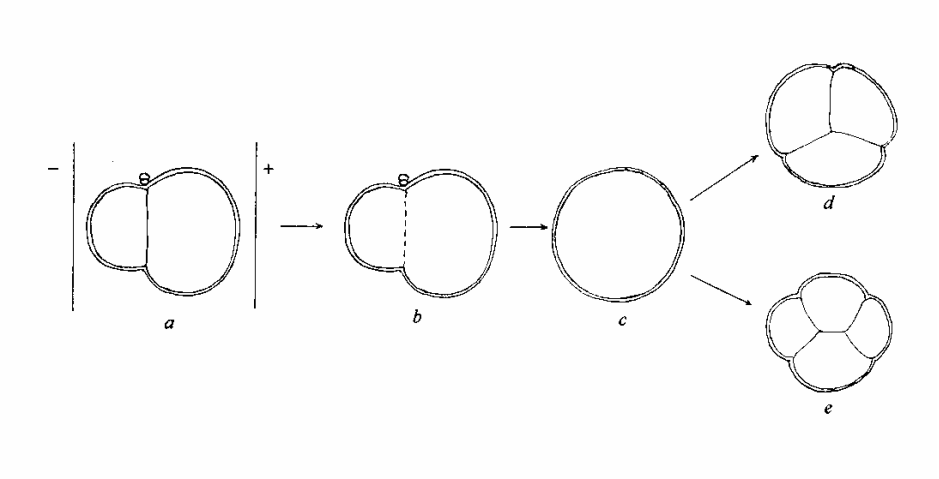

Fig. 4. Events following the application of an electric pulse to a cleaving oyster embryo: (a) 2-cell embryo; (b) breaking of the membrane; (c) fusion of the 2 cells; (d) and (e) new division into 3 or 4-cell embryos.

After cytoplasmic fusion, the behaviour of the two nuclei, one from each blastomere, was monitored. No consistent clear morphological feature, which could be used as a nucleus indicator, was found under the light microscope. However, two light spots (the presumptive nuclei) could sometimes be seen and in two of the fourteen embryos that fused moved toward each other; but fusion of those two presumptive nuclei could not be determined. The re-division of the four fused embryos was abnormal, resulting in three to four cells after next cleavage (Fig. 4).

Thermal Shock and Heat + Caffeine Shock

Altogether fourteen experiments on tetraploid inductions with thermal or heat + caffeine shocks were conducted. At the time the shocks were applied the cleavage furrow of the first mitotic division appeared on some zygotes (less than 5 %).

In these experiments, treated eggs tolerated all shocks (2 °C, 32 °C, 34 °C, 35 °C, and 32 °C + caffeine shocks) well and divided normally (Table 1). However, the development of eggs treated with 34 °C, 35 °C, and 32 °C + caffeine shocks was delayed and less synchronised than the controls. Thus when control eggs entered the 8-cell stage, eggs in 34 °C, 35 °C, or 32 °C + caffeine treated groups were at the mixed stages of 1-cell, 2-cells and 4-cells.

Tetraploid induction was evident with eggs treated with 34 °C, 35 °C, and 32 °C + caffeine shocks. Flow cytometry analysis of 19 h old larvae revealed that tetraploids were produced in experiments in 1996 (Table 2; Fig. 5). The 34 °C and 35 °C shock treatments produced the same percentage of tetraploids, 18 % on average. The 34 °C thermal treatment for a longer duration (23 min), did not increase the tetraploid percentages (20%). Tetraploid induction in eggs treated with 2 °C or 32 °C shock was not effective; less than 10 % tetraploids were revealed by flow cytometry analysis, compared with about 5 % tetraploid in the controls.

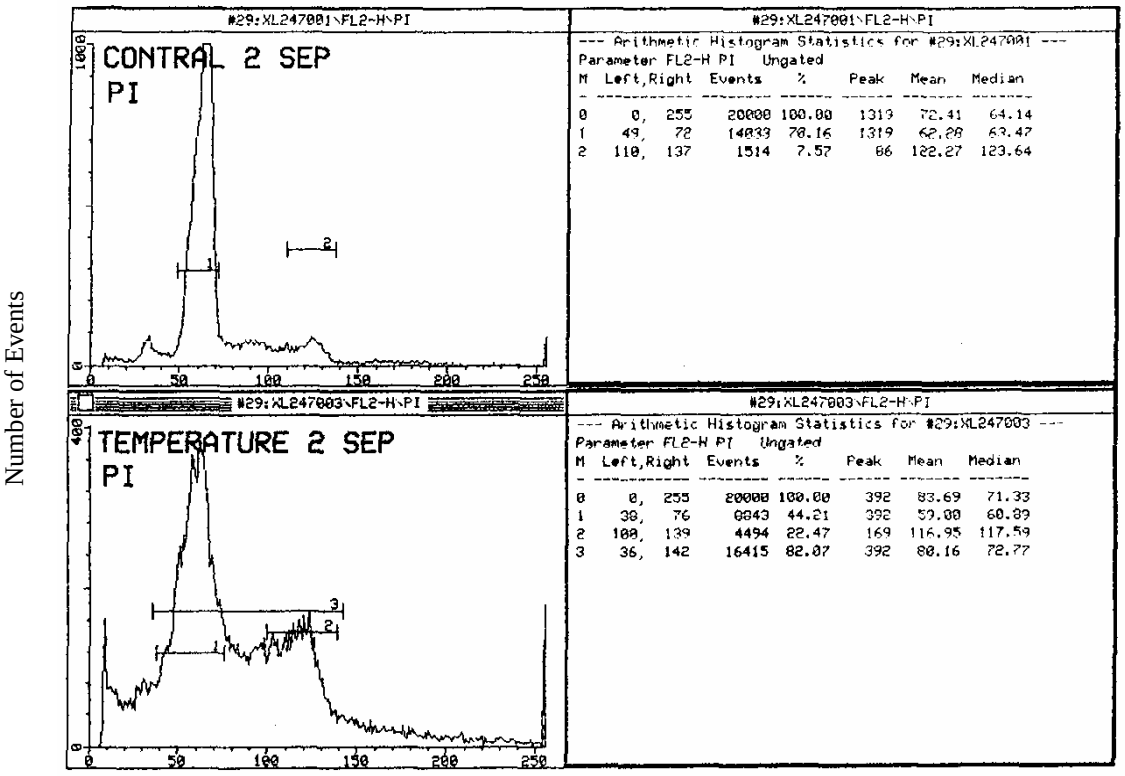

Fig. 5. Flow cytometric analysis report of 19 h old larvae from the experiment on inhibiting the first mitotic division with thermal shock. Upper: control larvae; Lower: thermal treated larvae. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

At 24 h post-fertilisation, D-shaped larvae were found in all treated groups. In treated groups there were a high percentage of embryos with abnormal development and many individual blastomere cells settled on the tank bottom.

In late 1997, the quality of eggs in both strip-spawned and naturally spawned batches was poor and their development after insemination was asynchronous. At 30 min post-fertilisation at 25 ^{o}C only 30 % of eggs were seen with polar body I. Whereas if good quality eggs were used more than 90 % should have at least one polar body at this time. Therefore, all six trials in December 1997 were abandoned at 2 h post fertilisation, although the experiments had been carried out as designed.

Table 1 Percentage of eggs surviving after thermal or heat + caffeine treatments*

Treatment	Percentage of eggs survived in control[1]	Percentage of eggs survived in treated group[2]
2°C	99	97
32°C	100	95
34°C	98	89
35°C	96	86
32°C + caffeine	98	83

*The treatments were applied at 55 min post-fertilisation at 25 °C. Samples were analysed at 8 h post-fertilisation.

1. The number of fertilised eggs from 100 randomly selected eggs, sampled 8 h post-fertilisation from the control group (a sub-sample of eggs from the same batch of eggs as the treatments).

2. The number of fertilised eggs that had more than 4 blastomeres from 100 randomly selected individuals, sampled from each treatment.

Table 2 Percentage of tetraploids induced by thermal or heat + caffeine treatments

Treatment	Number of times the experiments repeated	Tetraploid percentage at 19 hours[2]	
		average	highest
2 °C[1]	2	7.5	8
32 °C[1]	2	6.5	7
34 °C[1]	3	18	23
34 °C[3]	1		20
35 °C[1]	3	19	21
32°C + caffeine[1]	4	15	17

1. The treatments were applied at 55 min post-fertilisation for 15 min.

2. Results from ungated flow cytometry analysis.

3. The treatments were applied at 55 min post-fertilisation for 23 min.

Pressure Shock

There was one hydraulic pressure instrument available at the School of Biological Sciences, The Flinders University of South Australia, when this project was undertaken. As a result, replicated experiments with fertilised eggs from the same batch could not be conducted at the same time;

two trials were therefore conducted one after another, with an approximately 20-min interval between them. Most pressure experiments in this research project were carried out on a single batch of eggs (unreplicated).

The pressure equipment at Flinders University could not stabilise the high pressures for the duration required in these experiments. To overcome this, repeated additional pressure had to be added in order to maintain the required pressure(s). Thus, the pressure intensities used in this study consisted of two parts: an initial pressure and an extra pressure.

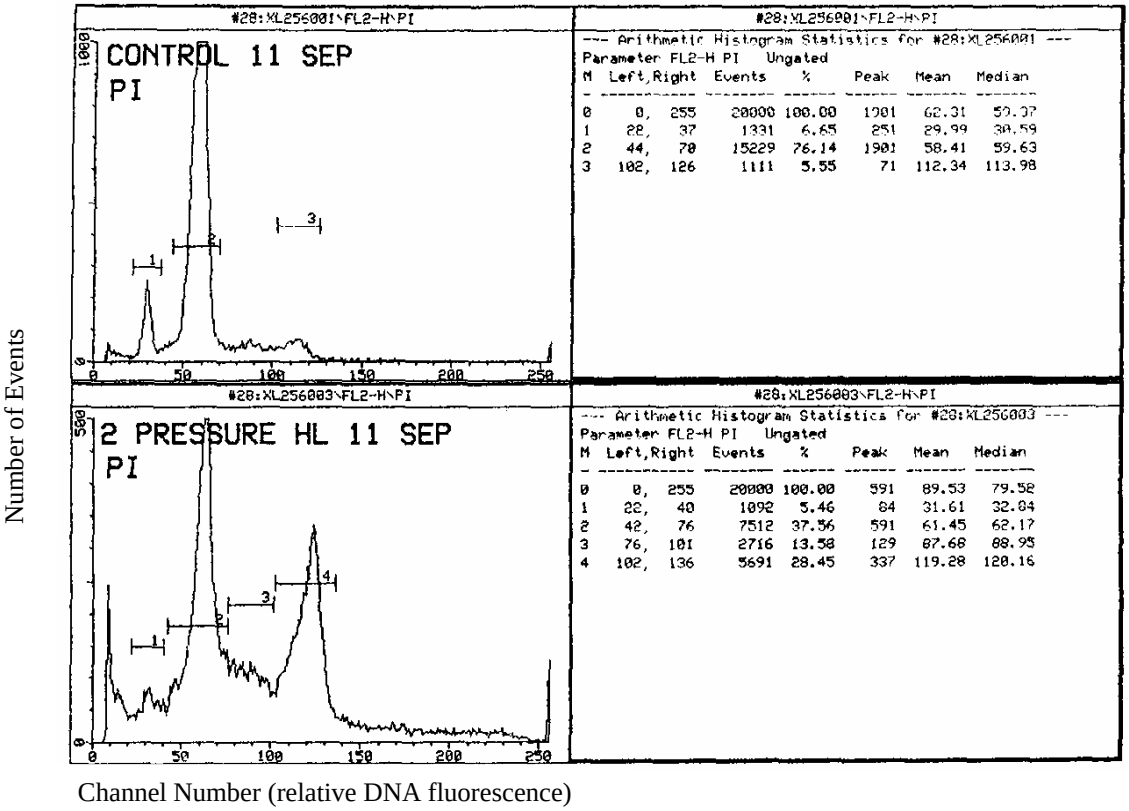

Fig. 6. Flow cytometric analysis report of 14 h old larvae from the experiment on inhibiting the first mitotic division with pressure shock. Upper: control larvae; Lower: pressure treated larvae. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

The percentages of tetraploid embryos induced by different hydraulic pressure treatments are shown in Table 3. The most efficient treatment was 550+20 kg/cm^2 pressure applied at 50 min post-fertilisation for a period of 10 min (Fig. 6). The treatment was not effective (less than 10 % tetraploids were verified by flow cytometry) when the treatment duration was reduced from 10 min to 5 min. At 50 min post-fertilisation the second polar body was released in more than 75 % of the fertilized eggs at 25 ^{o}C. Tetraploidy induction with pressure intensities less than 400+20

kg/cm^2 was not effective; less than 10 % tetraploids were produced from these treatments. Tetraploidy induction was evident at the intensity of 400+20 kg/cm^2, in both 10 min and 15 min treatments (Table 3). There was no difference in tetraploidy yields between these two treatments. A low level of tetraploids was also produced in the 550+20 kg/cm^2 treatment for 10 min, when treatments were applied 45 or 65 min post-fertilisation (Table 4). When the pressure intensity was increased to 600+20 kg/cm^2 the tetraploid yield dropped to 23 %.

Table 3 Percentage of tetraploids induced by hydraulic pressure treatments[1]

Treatment	Number of times the experiments repeated	Tetraploid percentage at 14~20 hours[2]		Percentage of eggs fertilised[3]	Percentage of eggs survived[4]
		average	highest		
300+20 kg/cm²					
10 min	2	9.5	10	98	98
350+20 kg/cm²					
10 min	2	8	10	100	98
400+20 kg/cm²					
5 min	1	-	10	99	97
10 min	2	13	13	100	96
15 min	2	13	14	98	96
450+20 kg/cm²					
10 min	1	-	21	100	93
500+20 kg/cm²					
10 min	1	-	21	98	89
550+20 kg/cm²					
5 min	1	-	9	98	90
10 min	1	-	29	100	86
600+20 kg/cm²					
10 min	1	-	23	98	53

1. The treatments were applied at 50 min post-fertilisation at 25°C.

2. Results from ungated flow cytometry analysis.

3. The number of fertilised eggs in 100 randomly selected eggs from the control group (a subsample of eggs from the same batch of eggs as the treatments). Samples were analysed at 8 h post-fertilisation.

4. The number of embryos that had more than 4 blastomeres from 100 randomly selected individuals, sampled from each treatment. Samples were analysed at 8 h post-fertilisation.

Table 4 Percentage of tetraploids produced by 550+20kg/cm^2 pressure treatments*

Initiation time (after fertilisation 25 °C)	Tetraploid percentage at 14~19 hours[1]	Number of experiments
45 min	14	1
50 min	29	1
55 min	21	2
60 min	19	1
65 min	11	1

* 10 min duration was used for all treatments.

1. Results from ungated flow cytometry analysis.

Except in the experiment in which 600+20 kg/cm^2 (about 8550 psi) was used to induce tetraploids, the eggs exposed to the shocks less than 600+20kg/cm^2 tolerated the treatments well, with more than 85 % of eggs dividing more than two times at 8 h post-fertilisation (Table 3). However, the treated eggs did not develop as synchronously as in the controls, and their development was delayed by at least one division cycle. At 24 h post-fertilisation, D-stage larvae were found in all treated groups, although abnormal development was high in groups treated with pressures higher than 450+20 kg/cm^2.

As in the thermal and heat + caffeine experiments in 1997, the eggs used in 1997 pressure trials were of poor quality, with unsynchronised development and only 30 % of eggs observed with polar body I at 30 min post-fertilisation. As a result of this, all fourteen trials with hydraulic pressures in December 1997 were abandoned 2 h post-fertilisation.

Chemical Shocks

Suppression of both polar body formations

The CB shocks were applied 7 min post-fertilisation, before the formation of the first polar body, and released after a period of 35 or 38 min (42 or 45 min post-fertilisation) at 25 °C. At this time, more than two thirds of the eggs in the controls already had released the second polar body, as viewed under the microscope.

Table 5 Percentage of tetraploids produced by suppression of both polar body formations with cytochalasin B (CB)*

Treatment duration	CB concentration	Tetraploid percentage at 14~19 hours	Date of the experiment
35min	1mg/L	35[2]	23 Sept. 1995
35min	0.5mg/L	<10[1]	13 March 1996
38min	0.5mg/L	<10[1]	13 March 1996

*All treatments were started at 7 min post-fertilisation.

1. Results from ungated flow cytometry analysis.

2. Results from direct chromosome count analysis.

The percentage of tetraploids produced in different trials was variable (Table 5). The most effective trial (September 1995) produced 35 % tetraploid oyster embryos, identified by direct chromosome count. In other trials the yields were mainly pentaploids, with the tetraploid peak not becoming clearly evident on the histograms of fluorescent values obtained by flow cytometry (Fig. 7).

The eggs in treated groups developed slowly and less synchronously than the control eggs. The embryos in this experiment (September 1995) had a very low survival rate, 0.1 % survival to day 2 in the control, resulting in limited larvae across all treatments. Consequently, these experiments were terminated.

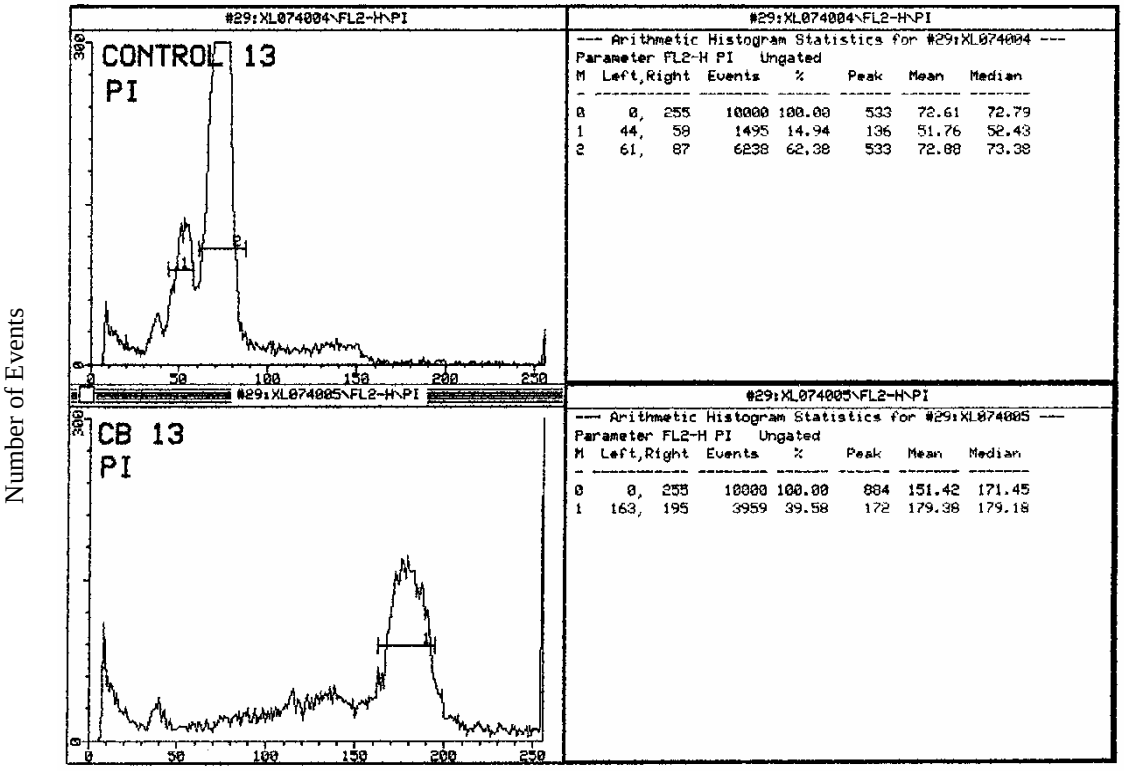

Fig. 7. Flow cytometric analysis report of 14 h old larvae from the experiment to inhibit both the first and second meiotic divisions with CB. Upper: control larvae; Lower: larvae treated with CB. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

Suppression of polar body I formation

The CB treatments were applied 5 min post-fertilisation at 25 °C or 15 min post-fertilisation at 18 °C, before eggs had formed the first polar body, and were released 15 or 20 min later. At this stage, more than two thirds of the zygotes in the control had already formed polar body I. Overall, the tetraploid yields following treatment were very low (<10 %, Table 6). At 14 to 19 h post-fertilisation, dissociated embryos in treated groups (18 and 25°C respectively) consisted primarily of diploid euploids or a mixture of diploid and triploid euploids, 2.5n and 3.5n aneuploids and other aneuploids of vicinities as analysed by flow cytometry (Fig. 9).

Table 6 Percentage of tetraploids produced by suppression of polar body I formations with cytochalasin B (CB) *

Treatment duration	Temperature	Tetraploid percentage at 14~19 hours[1]	Number of times the experiments repeated
15min[2]	18 °C	<10	2
15min[3]	25 °C	<10	4
20min[3]	25 °C	<10	1

*A concentration of 0.5mg/L cytochalasin B in 0.05% DMSO was used in all experiments.

1. Results from ungated flow cytometry analysis.

2. Treatments were applied from 15 min to 30 min post-fertilisation.

3. Treatments were started 5 min after fertilisation.

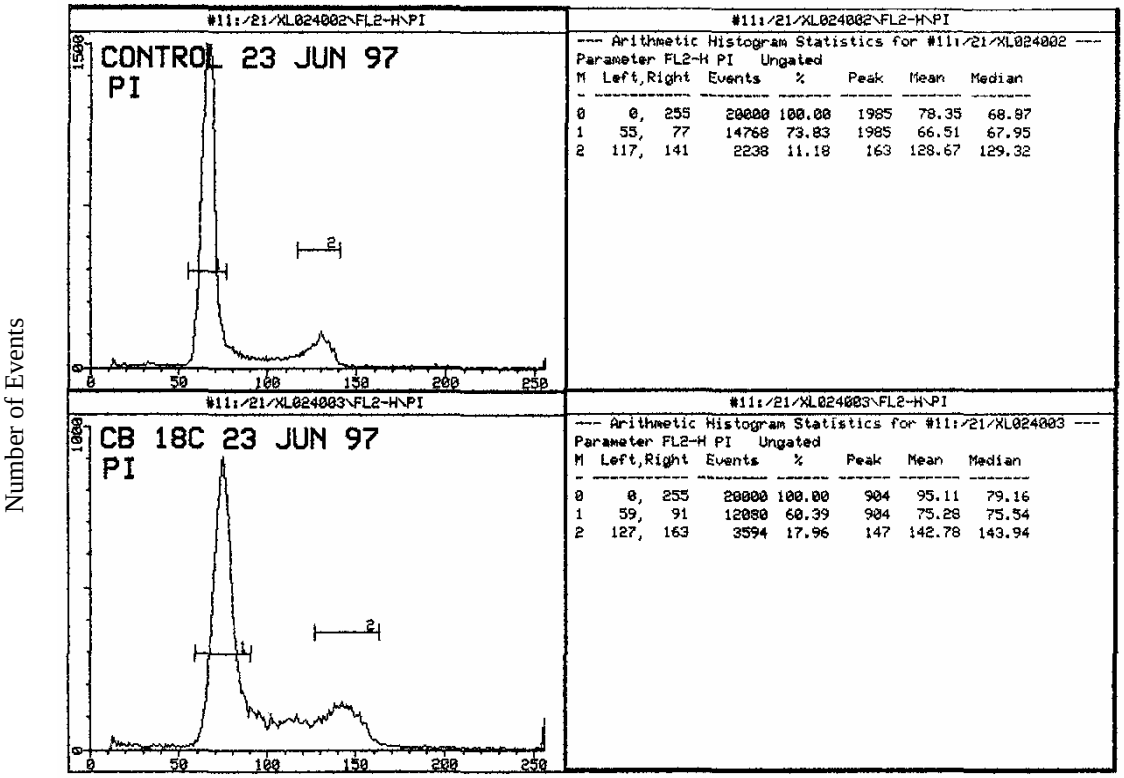

Fig. 8. Flow cytometric analysis report of 14h old larvae from the experiment on inhibiting the first meiotic division with cytochalasin B (CB) at 18 °C. Upper: control larvae; Lower: larvae treated with CB. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

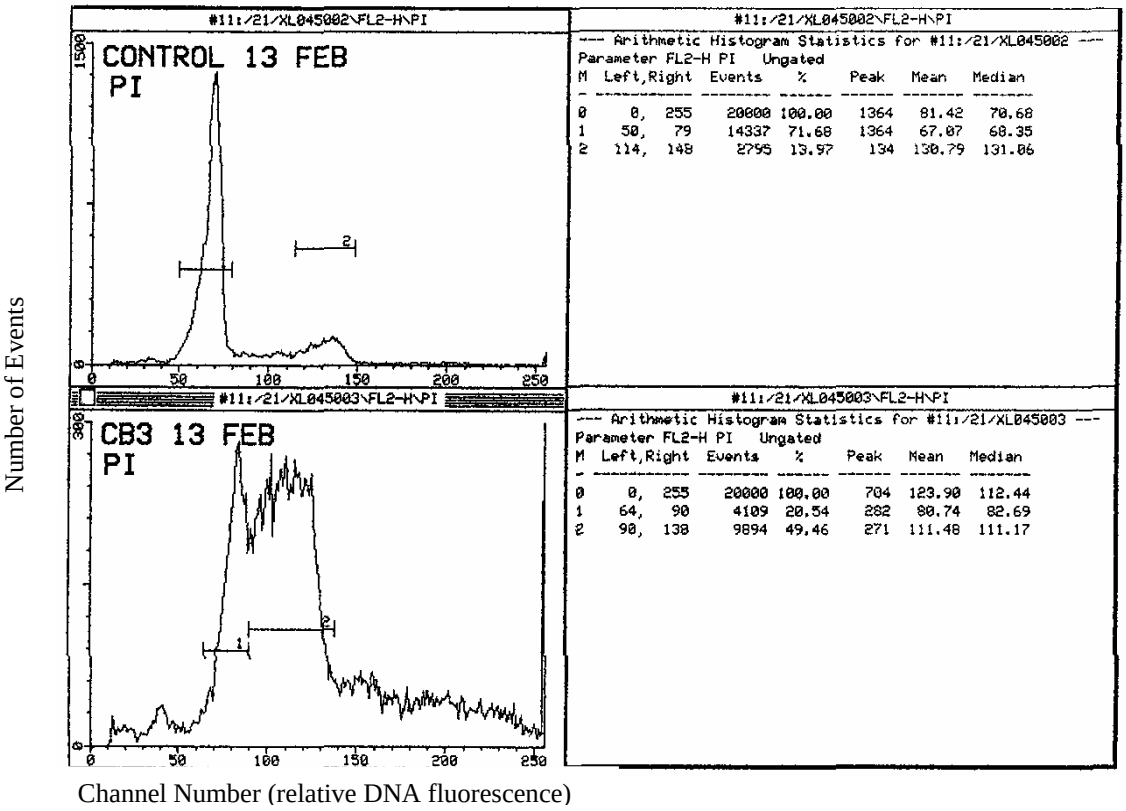

Fig. 9. Flow cytometric analysis report of 14 h old larvae from the experiment on inhibiting the first meiotic division with cytochalasin B (CB) at 25 °C. Upper: control larvae; Lower: larvae treated with CB. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

Suppression of the first mitotic division

The treatments were started when more than two thirds of the eggs had developed polar body II, which was usually at 45 to 50 min post-fertilisation at 25 °C. The treated eggs were then washed 20 to 25 min later, once over 80 % of the eggs in the control were at the 2-cell stage.

At 14 to 19 h post-fertilisation, the detergent dissociated cells of the treated groups were analysed by flow cytometry; the resulting histograms showed that these cells were primarily diploids and aneuploids, with no tetraploid cell populations evident (Table 7; Fig. 10).

Direct observation of the zygotes under the microscope revealed that the dosages of both CB and 6-DMAP used in this study were high enough to prevent the first mitotic division in Pacific oysters. The eggs did not divide in the presence of the CB, furthermore, the faint trace of the first cleavage furrow on some faster developed eggs disappeared a few minutes after eggs were immersed in the chemical. If treated eggs were washed before the control eggs started the second mitotic division, division resumed. However, instead of dividing from 1-cell to 2–cells, most

treated eggs formed two cleavage furrows at the same time, resulting in 3 to 4-cell embryos. The division was also delayed by about 10 min.

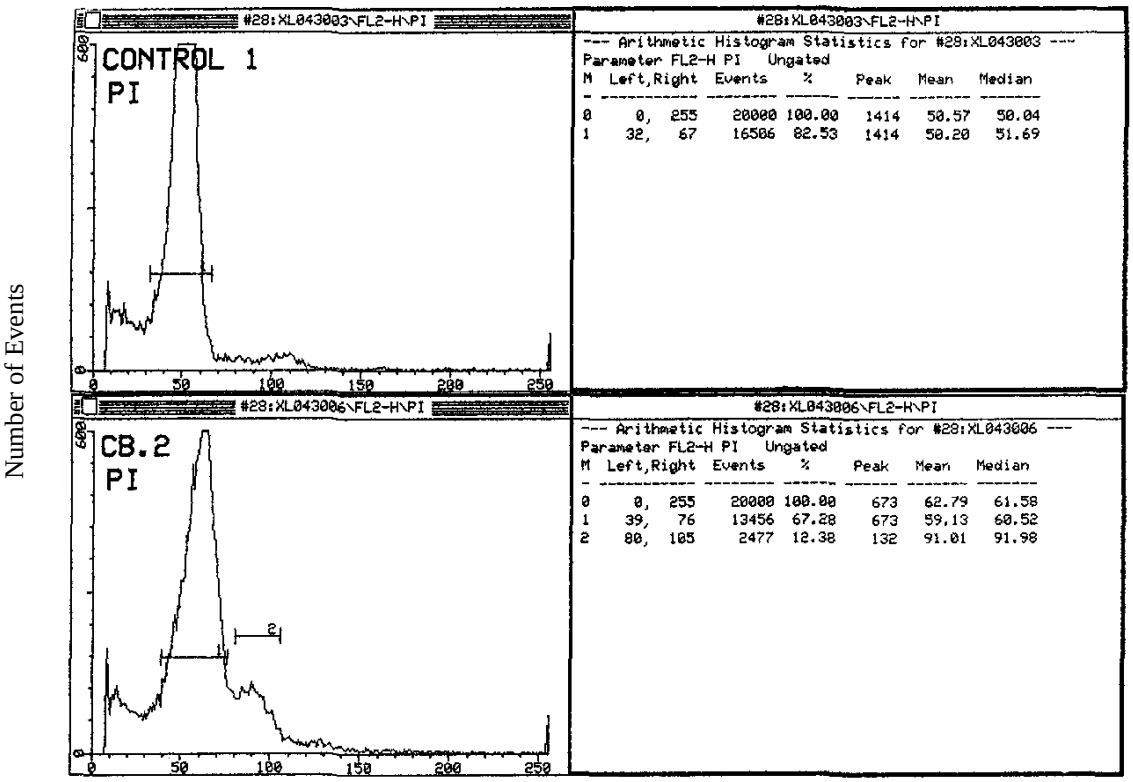

Fig. 10. Flow cytometric analysis report of 14 h old larvae from the experiment on inhibiting the first mitotic division with CB. Upper: control larvae; Lower: larvae treated with CB. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

Table 7 Percentage of tetraploids produced by suppression of the first mitotic division with cytochalasin B (CB) or 6-dimethylaminopurine (6-DMAP)*

Chemical	Chemical concentration	Tetraploid percentage at 14~19 hours[1]	Number of times the experiments repeated
CB[2]	0.5mg/L	<5	2
CB[3]	0.5mg/L	<5	3
6-DMAP[2]	450μmol/L	<5	2
6-DMAP[3]	450μmol/L	<5	2

*All experiments were conducted at 25 °C.

[1.] Results from ungated flow cytometry analysis.

[2.] Treatments commenced 45 min post-fertilisation, for a period of 25 min.

[3.] Treatments commenced 50 min after fertilisation, for a period of 20 min.

Tetraploidy inductions by combined chemicals

The toxicity of 2-mercaptoethanol, the chemical that was first introduced into chromosome manipulation in this research, was initially tested in blue mussel *Mytilus edulis*. The eggs survived the treatment well. Eggs were treated for a period of 30 min, with a dose rate twice the concentration of 2-mercaptoethanol used in a later experiment. More than 95 % of the eggs treated resumed division and developed into normal larvae after being moved from the chemical solution. These larvae were cultured in a recirculating aquarium system at Flinders University. Larvae developed through metamorphosis and grew into 2 cm normal mussels after one year, despite not being fed to satiation.

Pacific oyster eggs were exposed to the chemicals (2-mercaptoethanol and CB) a few minutes post-fertilisation and removed when more than two thirds of the eggs in the control had developed the second polar body.

Tetraploids in combined chemical trials were analysed by either direct chromosome count or flow cytometry analysis. Normally approximately 20 % tetraploids were found at 14~19 h post-fertilisation (Fig. 11; Table 8) in treated group(s), if eggs developed synchronously in control(s). The highest percentage of tetraploids obtained from these treatments, 46 %, was on day 1 of the trial conducted on 29 January 1997 (Fig. 12). A similar result was also obtained in the trial on 12 March 1997 (Fig.13). The ploidy status presented in this report was an approximate percentage based on ungated flow cytometry data. If gated data was used in the analysis, the tetraploid

percentages should be higher. For instance, if background data were removed in Fig. 12, the tetraploid percentage would be much higher than 50 %.

Table 8 Percentage of tetraploids induced by combined chemical treatments*

Year(s)	Number of times the experiments repeated	Tetraploid percentage at 14~19 hours		Date of the highest tetraploid induced
		normal	highest	
1995/1996	39	~20[1]	35[2]	23 Feb. 1996
1996/1997	37	~20[1]	46[1]	29 Jan. 1997
1997[3]	10	NA	NA	NA

*The treatments were applied a few min post-fertilisation at 25 °C.

1. Results from ungated flow cytometry analysis.

2. Results from direct chromosome count analysis.

3. All experiments were terminated 2 h post-fertilisation because of highly asynchronous development of the fertilised eggs.

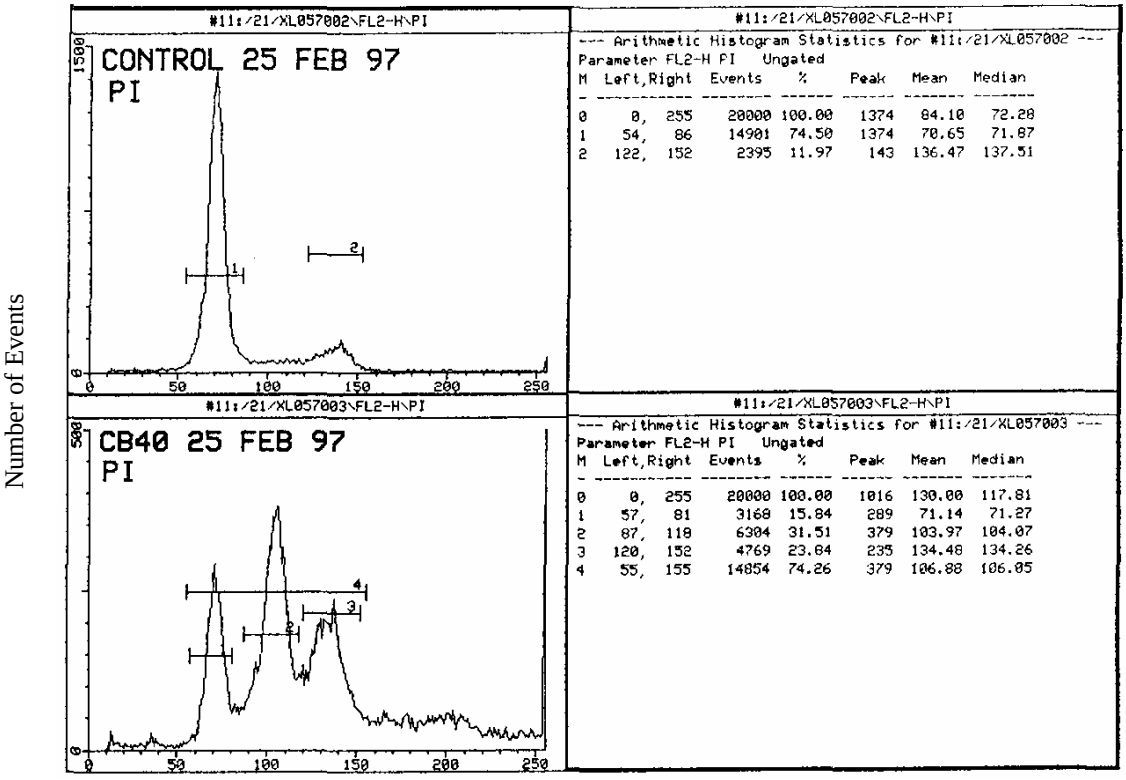

Fig. 11. Flow cytometric analysis report of 14 h old larvae from the experiment on inhibiting both the first and second meiotic division with combined chemicals. Upper: control larvae; Lower: larvae treated with combined chemicals. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

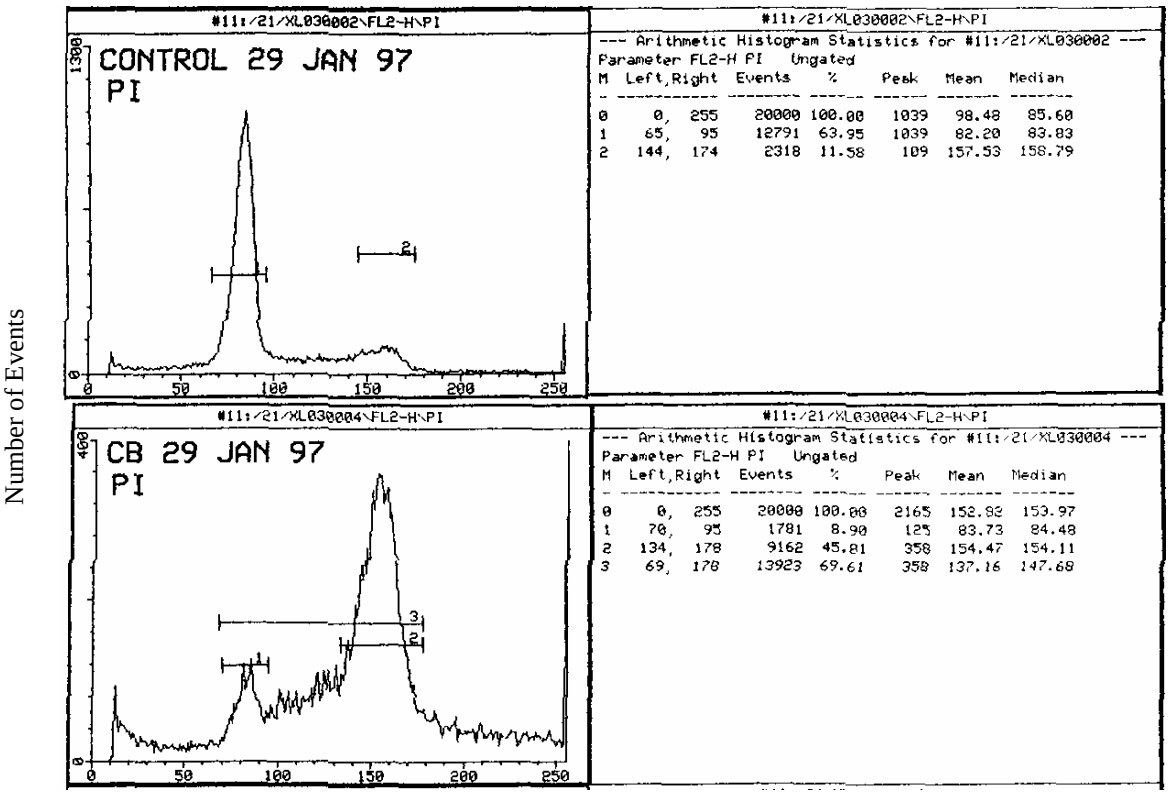

Fig. 12. Flow cytometric analysis report of 14 h old larvae from the experiment on inhibiting both the first and second meiotic division with combined chemicals. Upper: larvae treated with combined chemicals; Lower: control larvae. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

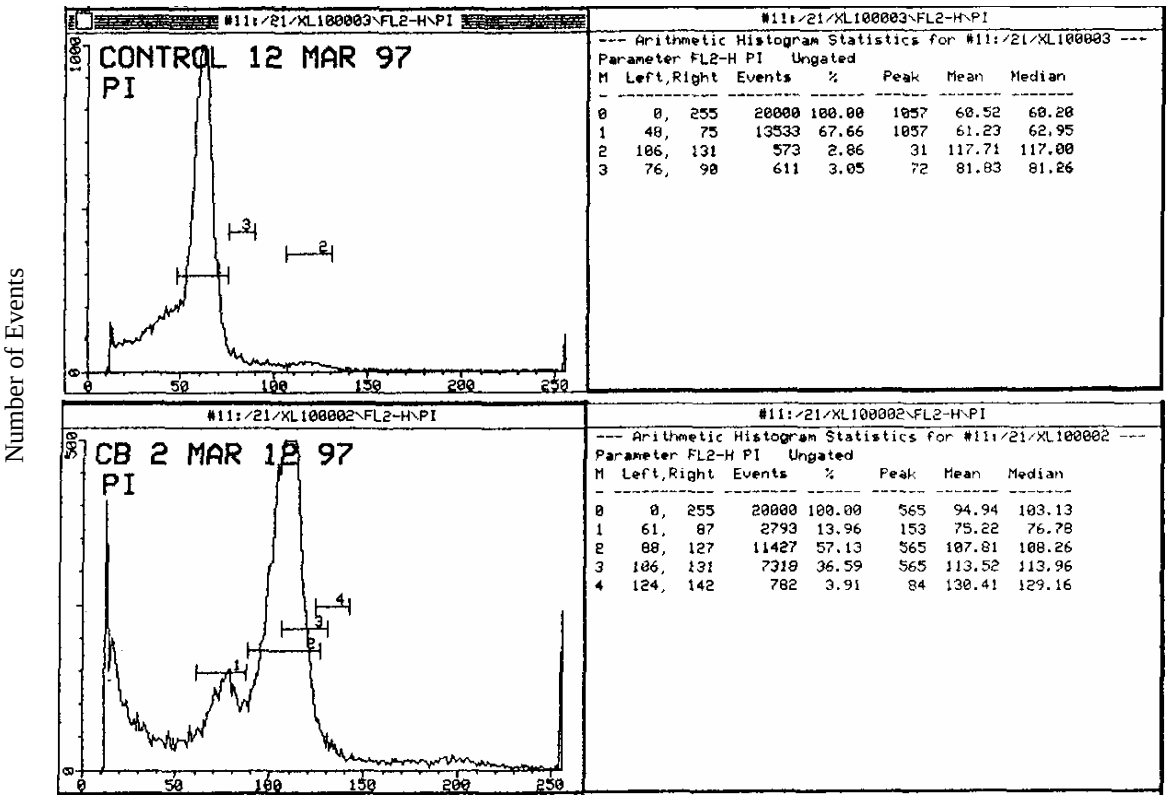

Fig. 13. Flow cytometric analysis report of 14 h old larvae from the experiment on inhibiting both the first and second meiotic division with combined chemicals. Upper: larvae treated with combined chemicals; Lower: control larvae. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

Pacific oyster eggs tolerated the treatments well, with approximately 90 % of fertilised eggs in the treated group dividing more than two times at 8 h post-fertilisation. However, the addition of chemicals to treatments interrupted the fertilisation success of eggs if they were at different maturation stages when the sperm was added. The treatments also resulted in heavy mortalities during the larval stages (Table 9), with some batches having no larvae survive beyond metamorphosis.

Table 9 The fertilisation and survival rates of Pacific oysters at different developmental stages in the two combined chemical experiments*

Date of experiment	23 Feb. 1996		25 Feb. 1997	
	Control [d]	Treated	Control [d]	Treated
No of females	1	same as control	4	same as control
No of males	1	same as control	3	same as control
No of eggs used	$10X10^4$	$25X10^4$	$2.8X10^6$	$6.3X10^6$
Fertilisation rate (%)	99 ($9.9X10^4$)	99 ($24.75X10^4$)	95 ($2.66X10^6$)	85 ($5.36X10^6$)
Survival				
D-stage larvae at 30 hours PF (%)[a]	98 ($9.7X10^4$)	63 ($15.59X10^4$)	94 ($2.50X10^6$)	53 ($2.84X10^6$)
Total number of larvae before metamorphosis[b]	$2.0X10^4$ ($8.0X10^4$)[f] (80.8%)	$4.5X10^4$ (18.2%)	$0.03X10^6$ ($1.80X10^6$)[f] (67.7%)	$0.36X10^6$[e] (20.1%)
Total number of spat set	$1.2X10^4$ (60%)	$0.43X10^4$ (9.6%)	$0.018X10^6$ (60%)	$0.007X10^6$[e] (1.9%)
Total number of oysters at 10 to 12 months	800 (5976)[f] (49.8%)	350 (8.1%)	1500 (10998)[f] (61.1%)	2100 (30%)

* Tetraploidy spats from these two experiments were confirmed by flow cytometry analysis.

[a] Percentage of D-stage larvae post-fertilisation (PF).

[b] Total number of larvae at the date when first metamorphosis was induced by epinephrine. Larvae in the controls were randomly culled off in 1996 (75 %) and 1997 (98 %), prior to the first metamorphic induction.

[c] Oyster numbers at the time when the ploidy status of individual animals was analysed by flow cytometry.

[d] The larval and spat densities in controls had randomly been reduced at different stages, to maintain similar densities as in the treated groups.

[e] Two thirds of 8-day old larvae were sent to South Australian Oyster Hatchery at Port Lincoln. Heavy mortality occurred two days before the first metamorphosis was induced at Flinders University.

[f] The theoretical numbers if random culling off were not conducted in controls.

The fertilisation and survivals rates of Pacific oysters at different developmental stages of the two experiments are shown in Table 9. In the experiment conducted in 1996, there were no eggs found with a germinal vesicle when insemination started, as observed under a microscope. The

percentage of eggs fertilised was high, 99 %, in both the chemical treated group and the control. In the experiment conducted in 1997, approximately 12 % of the eggs were still at germinal vesicle stage when eggs were inseminated by strip-spawned sperm. The fertilisation rate in the treated group was 10 % lower than that in the control group. The percentage of fertilised eggs that developed into the D-stage larvae was much lower in the treated groups than in the controls: 63 % and 53 % in treated groups, and 98 % and 94 % in controls, in 1996 and 1997 respectively. Larvae in the treated groups were then stocked at densities of 1-15 larvae/mL at different stages before metamorphosis, while the larvae in the controls were randomly culled three times to maintain similar densities as in the treatment groups. In 1996, 75 % of the larvae in the control group were culled off, while in 1997, 98% were culled prior to settlement. In 1997, 66 % of 8-day old larvae in the treated group were sent to the South Australian Oyster Hatchery in Port Lincoln. The developmental data of oysters after the D-stage presented in this report was from Flinders University, as there was no control group at the South Australian Oyster Hatchery.

For the trial conducted in 1996, approximately 80.8 % of fertilised eggs or D-stage larvae in the control group developed to eyed larvae, of which 60 % survived metamorphic treatment and developed into normal spat; while in the treated group, only 18.2 % of fertilised eggs or 28.9 % of D-stage larvae survived to the stage near metamorphosis, from which 9.6 % of spat were obtained. The eggs/larvae in the 1997 trial had similar survival rates as the controls in the 1996 trial at the different developmental stages recorded; 67.7 % of fertilised eggs and 72 % of D-stage larvae developed to metamorphic larvae, of which 60 % developed to the spat stage. Although the percentage of the fertilised eggs and D-stage larvae that developed to the metamorphic stage in the treated group in 1997 (20.1 %) was slightly higher than in 1996 (18.2 %), only 1.9% of metamorphic larvae grew through to the spat stage; heavy mortality occurred two days before the first artificial induction of cultchless oysters was applied. In both years, the percentage of spats in the treated groups that survived to 10 to 12 months, was very low; 8.1% in 1996 and 30.0% in 1997; while the survival rate in the controls was higher, 49.8 % in 1996 and 61.1 % in 1997. The improvement in the performance of spat in 1997 may be due to the use of the newly designed recirculating system at Flinders University.

The ploidy status of twenty-eight and seventy-nine 10 to 12 months old spats were analysed by flow cytometry for 1996 and 1997 trails respectively and one individual was confirmed as tetraploid in each year cohort (Fig 14, Table 10). Mosaics were also found in both years oysters (Fig 15, Table 10).

Table 10 Percentage of tetraploids in Pacific oysters at different development stages in two experiments*

Experiment date	Day 1	Day 5	Day 15~20	10~12 months
23 Feb. 1996	35[2]	30[1]	31[1]	1 in 28[1]
25 Feb. 1997	24[1]	8[1]	N/A (heavy mortality at Flinders University)	1 in 79[1]

*Combined chemical treatments were applied.

[1.] Results from ungated flow cytometry analysis.

[2.] Results from direct chromosome count analysis.

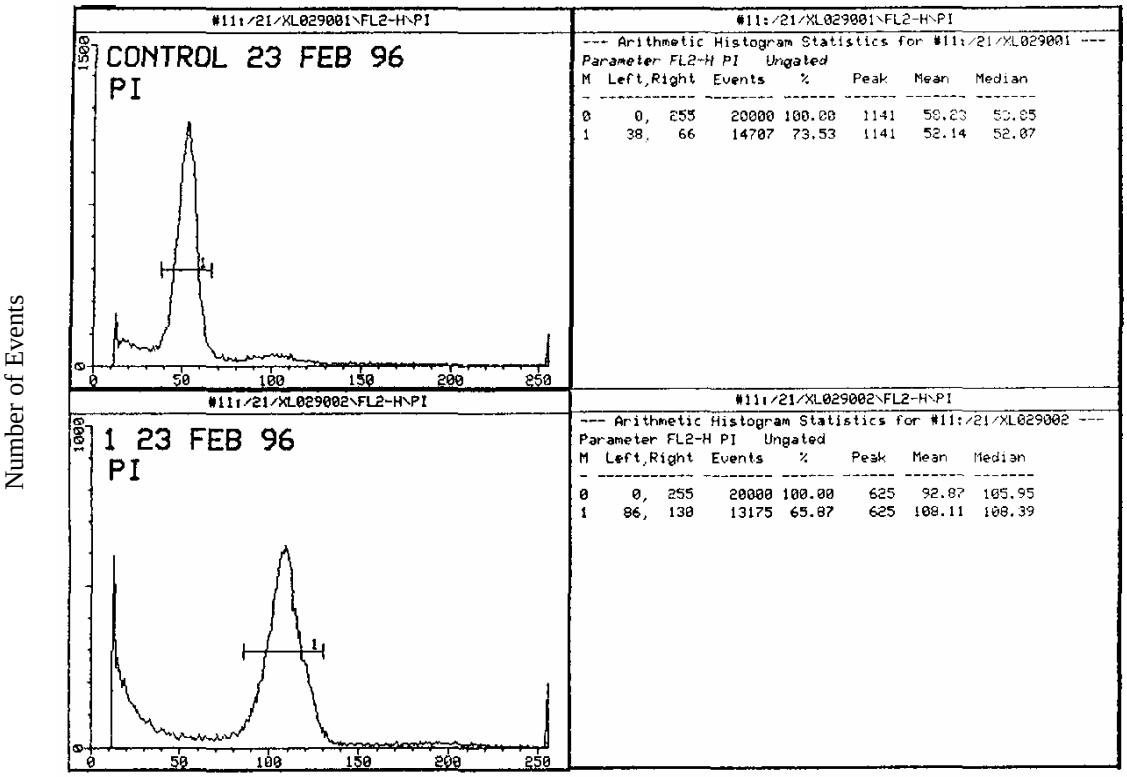

Fig. 14. Flow cytometric analysis report of 10 month old spat from the experiment on inhibiting both the first and second meiotic division with combined chemicals. Upper: control larvae; Lower: larvae treated with combined chemicals. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

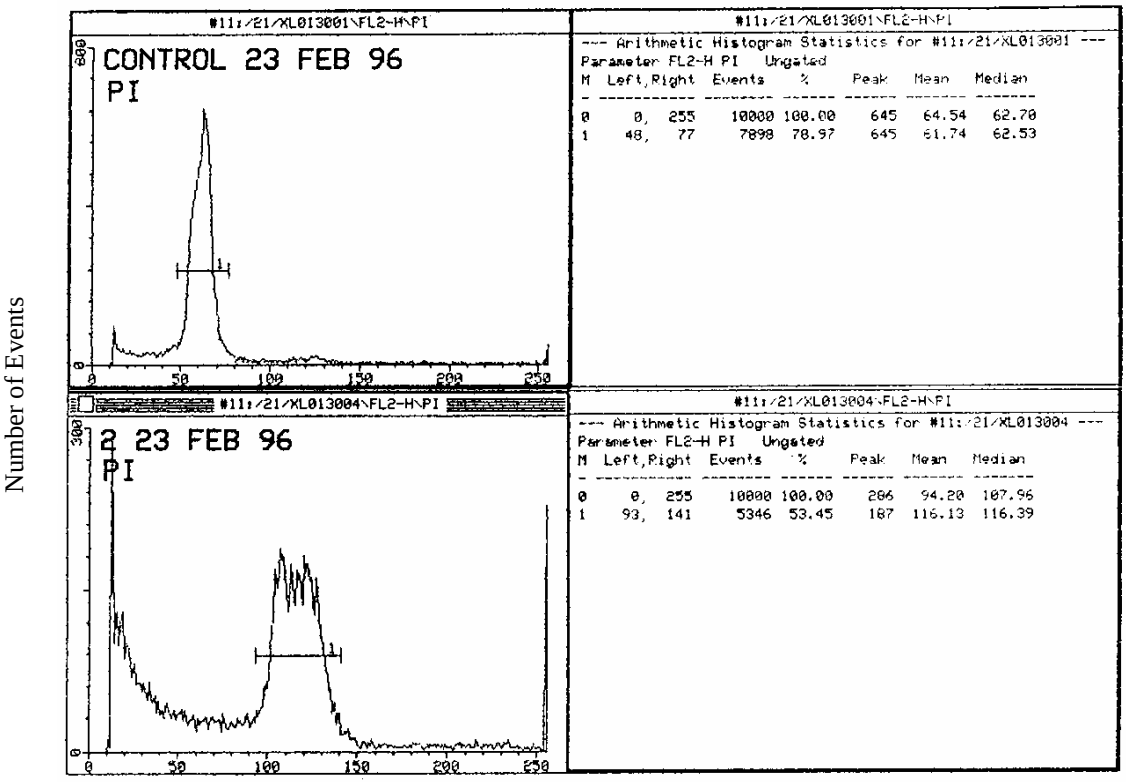

Fig. 15. Flow cytometric analysis report of 10 month old spat from the experiment on inhibiting both the first and second miotic division with combined chemicals. Upper: control larvae; Lower: larvae treated with combined chemicals. The numbers in column M on the right are the IDs for the channel ranges, which are marked on the graphs on the left.

DISCUSSION

TETRAPLOID INDUCTIONS

Electrofusion

The experiments described here focussed on the 2-cell embryonic stage in order to increase the probability of tetraploid induction. After applying electrical pulses in these trials, the two cells of some 2-cell stage embryos, with their mutual longitudinal axis perpendicular to the electrodes, were clearly fused. However, re-division of the fused embryos monitored in our experiment was abnormal, resulting in three or four cells after the next cleavage. A possible explanation for this could be that the fusion of the two blastomeres produced a hybrid cell, with two separated nuclei that failed to form a common metaphase plate and have an incorrect organisation of the microtubule system (Cadoret 1992); alternatively, the two separated nuclei of a hybrid cell could form a common metaphase plate, with a multi-polar spindle, allowing the cells to divide into more than two cells (Li 1996; Li and Chen 1996).

The 0.6 M sucrose solution, a non-electrolyte solution, which was used to minimise the effects of heat from electric fields, has been shown in these experiments and studies by Cadoret (1992) as having no detrimental effects on the development of oyster embryos. Embryos were found to continue normal development after having been immersed in the solution for about half an hour.

In tetraploid induction experiments, Cadoret (1992) applied the electrical pulses to embryos prior to completion of the first cell division and produced up to 20 % and 26 % tetraploids in oysters and mussels, respectively. However, the direct chromosome count method used in that study to estimate ploidy levels, could not differentiate tetraploid embryos from tetraploid/diploid/triploid mosaics, because only one metaphase spread from one cell is normally analysed for an individual embryo.

Cadoret (1992) suggested that the electrofusion method for increasing tetraploid induction could be improved by orientating the 2-cell embryos perpendicularly to the electrodes, before delivering electrical pulses. Such an orientation could be achieved by di-electrophoresis; applying a highly non-homogeneous and alternating electric field (Zimmermann 1987). In addition to these, techniques in controlling the activities of the microtubular organising centre should also be addressed, as multi-polar mitotic division will disrupt normal embryogenesis.

Thermal and heat + caffeine shocks

Significant levels of tetraploids were induced in this study, by suppressing the first mitotic division with the use of thermal, and heat + caffeine shocks. The highest percentage of tetraploids produced was 23 %, which is lower than the best yield (45 %) produced by Guo *et al.* (1994) in the same species with heat shocks. In the study by Guo *et al.* (1994), tetraploid percentage was analysed using the direct chromosome counting method, whereas ungated flow cytometry analysis was used in this study. Results from ungated flow cytometry analysis are much lower than the gated flow cytometry analysis and direct chromosome counting because events from debris are also included in the ungated analysis.

The tetraploids produced in Pacific oysters with both thermal, and heat + caffeine shocks are much lower than the percentages for other animals, such as newts (94 %, Fischberg 1958). The failure to induce higher levels of tetraploids in oyster eggs treated with heat shocks, was explained by Guo *et al.* (1994) as the result of unsynchronised egg development in Pacific oysters, which is greatly affected by the female condition and other environmental factors. A similar explanation was also used to justify the unreliable production of triploids by direct physical or chemical treatment of zygotes, produced by crossing diploid broodstock (Downing and Allen 1987).

In contrast to experiments conducted by Guo *et al.* (1994), in which the longer heat shock at a lower temperature produced more tetraploids than shorter shocks at a higher temperature, the trials with the 34 ^{o}C treatment for a longer duration (23 min) in this study did not increase the tetraploid percentage. This may be due to the narrow window of opportunity that is available for effective suppression of the first mitotic division using heat shock. The differences between studies might also suggest that the optimum shock conditions (temperature and duration) may not be universally applicable to all strains or environments and may need to be fine tuned for new situations.

Tetraploid induction in eggs treated with a 2 ^{o}C cold shock was not effective, with less than 10 % tetraploids revealed by flow cytometry analysis. This may be due to two factors: the unsynchronised development of treated eggs; and the possibility that the cold shock conditions used in this research may not be effective in blocking the first mitotic division in Pacific oysters at all. In experiments on triploid induction using cold shock, it was found that the cold shock treatment was most effective when it was initiated just before polar body II extrusion (Yamamoto and Sugawara 1988). The timing of shocks with respect to egg development seems more critical in cold shock treatments than in heat shock treatments.

Both caffeine and heat shocks are known to abolish cell spindle and inhibit their cleavage (Yamamoto *et al.* 1990); their synergistic actions were observed in experiments on triploid inductions with mussels and Pacific oysters (Yamamoto *et al.* 1990; Scarpa *et al.* 1994). The effect of caffeine on tetraploid induction in Pacific oysters was tested in this study by applying the treatment (32 °C + 15 mM caffeine) for a period of 15 min, 55 ~ 70min post-fertilisation. It yielded 10 % more tetraploids than a single heat shock (32 °C), which is similar to the tetraploid levels produced by single heat shocks at higher temperatures (34 °C and 35 °C). However, unlike triploid induction in mussels and oysters, in which the combined shock resulted in both higher yields and larval survival, the use of a caffeine + heat shock at a relatively low temperature slightly reduced larval survival in tetraploid induction in this study.

Pressure shock

Hydrostatic pressure shock has previously been successfully used in inducing triploidy in molluscs, such as Pacific oysters, abalone, scallops and pearl oysters (Allen *et al.* 1986; Arai *et al.* 1986; Komaro and Wada 1989; Shen *et al.* 1993). The highest triploid yield was 76 %, which was obtained by Shen *et al.* (1993) in the pearl oyster. The optimum or sub-optimum pressures used in triploid productions were widely variable for different species; 200kg/cm^2 for abalone, scallops and pearl oysters (Arai *et al.* 1986; Komaro and Wada 1989; Shen *et al.* 1993); and approximately 550 kg/cm^2 for Pacific oysters (Allen *et al.* 1986). Experiments in this study revealed that the pressures (6000 ~ 8000psi = 420 ~560 kg/cm^2) suggested by Chaiton and Allen (1985) and Allen *et al.* (1986) for producing triploid Pacific oysters, are high enough to suppress the first mitotic division. Pressures higher than that were found to dramatically reduce survival rates. For example, in this experiment, survival at 8 h post-fertilisation dropped from 86 % to 53 %, when pressures were increased from 550 kg/cm^2 to 600 kg/cm^2.

In this study repeated additional pressure (20 kg/cm^2) was added to maintain the pressure required for the treatment. However, the effects of this pressure fluctuation are not clear.

Previous studies have shown that the optimum time to apply pressure shocks to induce ploidy manipulation in fishes and pearl oysters is during metaphase or early anaphase of meiosis or mitosis (Myers *et al.* 1986; Shen *et al.* 1993). In this study, the highest tetraploid yield was produced when 550+20 kg/cm^2 pressure was applied from 50 to 60 min post-fertilisation at 25 °C. This corresponds to the metaphase stage or the beginning of anaphase of the first mitotic division, and implies that the mechanism of tetraploidisation in Pacific oysters with pressure shock may be the same as that in fishes and pearl oysters.

Chemical shocks

Suppression of both polar body formation

The method used to suppress the formation of both polar bodies with chemical shocks was developed by Scarpa *et al.* (1993) in mussels, *Mytilus galloprovincialis*. Trials by Scarpa *et al.* (1993) produced 17 % tetraploid spats as discovered by DNA microfluorometric analysis. Similar techniques have been attempted on Pacific oysters in this study. The tetraploidy levels at the larval stage ranged from undetectable by flow cytometry analysis, to 35 % by direct chromosome count. The trial that produced 35 % tetraploids was conducted in October 1995, in which the gametes were collected from laboratory conditioned broodstock. The eggs from these spawnings developed slowly and asynchronously following fertilisation, and had a very low survival rate (0.1 % at day 2 in the control group). As a result of this, limited larvae remained in the treatment groups. In other trials, the yields were mainly pentaploids (Fig. 7), which correspond to the theoretical ploidy level that would be achieved if both polar bodies were suppressed (*i.e.* four sets of maternal chromosomes and one set of paternal chromosomes result in a pentaploid nucleus). Unless this method can be modified and its role in tetraploid induction clarified, further attempts with this method cannot be justified.

Suppression of polar body I formation

The method to suppress polar body I formation was developed by Stephens and Downing in Pacific oysters at 18 ^{o}C. However, no details were available from their published abstract. This method was further tested in this study at both 25 ^{o}C and 18 ^{o}C. No promising tetraploid results were obtained from the seven experiments conducted. The poor results achieved in this study imply that this method may be population specific, or strongly influenced by the gametes conditions and using this method to produce tetraploids is therefore considered unreliable.

Suppression of the first mitotic division

Eggs in trials investigating suppression of the first mitotic division were found not to divide in the presence of CB, furthermore the faint trace of the first cleavage furrow on some faster developing embryos disappeared a few minutes after eggs were immersed in the chemical. This might be due to CB inhibiting the polymerisation of actin filaments, which play an important role in cytokinesis (Longo et al. 1993). Eggs that were removed from the chemical before the second mitotic division appeared in the control group, and washed, formed two cleavage furrows at the same time resulting in 3 or 4-cell embryos, rather than the expected 2-cell. This indicates that the CB treatment used in this study did inhibit cytokinesis (cell division), but did not interrupt the process of karyokinesis (segregation of chromosomes) or the microtubular organising centres'

activities. Chromosome manipulations with chemical treatments at the first mitotic division have also been attempted on fishes. Preliminary experiments using varying concentrations of mitotic inhibitors failed to induce chromosome doubling, however, diploid-tetraploid mosaics were produced in some species (Varadaraj and Pandian 1988). This indicates that CB might not be an effective method for suppressing the first mitotic division for tetraploid inductions.

Tetraploidy inductions by combined chemicals

In the present study, tetraploid Pacific oyster spats were produced in two groups of eggs that had been treated with CB and 2-mercaptoethanol at meiotic stages. Although tetraploid percentages at spat stages were low in both groups, tetraploids, tetraploid/triploid, and tetraploid/aneuploid mosaics were detected in the ten to twelve month old spat (Figs. 14 and 15). The relative DNA contents of tetraploid spat analysed by flow cytometry are twice that of diploid spat in the control, although, compared with the diploid histogram, tetraploids had a relatively wide peak.

Tetraploid genomes were probably formed from the joining of a triploid female genome and a haploid male genome. The chemical treatments may have contributed to the different gamete structures that were obtained, as the fertilisation success of eggs was considerably interrupted by treatments, especially when the eggs were at different maturation stages when the sperm was added. Detailed studies on chromosome behaviour and molecular changes in cell membranes in CB and 2-mercaptoethanol treated eggs will be needed to determine the mechanism of tetraploid induction in oysters.

Clear euploidy peaks shown in Figs. 11, 12 and 13 were typical of the combined chemical method. These results show the significant improvement in euploid levels in the combined chemical method, in comparison with using CB in isolation (Fig. 9). This indicates that "tripolar segregation" (Guo *et al.* 1992b) after blockage of polar body I release was significantly decreased, while "separated bipolar segregation" (Guo *et al.* 1992b) should be the main event for the treatments showed in Figs. 12 and 13. The results also indicate that this method should improve the efficiency of the techniques involved in blocking polar body I formation, such as the "blocking polar body I in eggs from triploids" tetraploid production method and the methods used for direct tetraploid induction in four other bivalve species: blue mussel (Scarpa *et al.* 1993); Manila clam (Allen *et al.* 1994); Zhikong scallop (Yang *et al.* 2000); and dwarf surfclam (Peruzzi and Guo 2002).

Heavy mortalities were found during the larval stages in all treated groups, with no larvae surviving beyond metamorphosis in some experiments. This may be because the resulting larvae were still predominantly aneuploid after treatment, despite the experiments being designed to

increase the euploid progeny level (triploid and tetraploid); or the eggs used in some experiments were too sensitive to the treatments. The tetraploid histograms of one-day-old larvae in this study support this notion, as they were found to be slightly less than two times the relative DNA content of diploids. This is similar to results found in the histograms of other species based on flow cytometric analysis and DNA microflurometry analysis, that were thought to be a result of aneuploidy (Allen and Stanley, 1983; Scarpa *et al.*, 1993; Fig. 12).

The tetraploid oyster spats in this study, as in studies on mussels (Scarpa *et al.* 1993), were detected from animals classified as small. Scarpa et al. (1993) suggests that the difficulties in separating the dead larvae from small animals and the likelihood of overlooking and losing small slow-growing spats, could explain why tetraploids were not found in earlier studies.

SURVIVALS OF TETRAPLOIDS

The poor survival of tetraploid oysters may be due to the problems associated with incomplete polyploid conversion. One such problem is the appearance of mosaics, which may result from mis-inheritance of extra microtubular organising centres, or chromosome losses during cell divisions. Larval or early spat stage mosaics could hardly be detected by the current ploidy verification methods used in oyster chromosome manipulation. Mosaics have been reported in fishes as an unwanted result of polyploid induction with heat, pressure and chemical treatments, especially with chemical agents such as CB (Allen and Stanley 1979). Mosaic individuals have been found in this study (Fig. 15).

Aneuploids are another problem that could be linked to the poor performance of treated larvae. An examination of the histograms produced from flow cytometric data revealed that many tetraploid histograms were relatively flat with a broad base, which might indicate a wide range in the number of chromosomes in different cells within an individual and in different individuals (Fig. 12). In contrast to this, histogram results from diploid control animals have a relatively narrow peak (Fig. 12). A similar phenomenon was observed by Allen and Stanley (1983) in tetraploid hybrid grass carp.

Another alternative explanation for poor survival in tetraploid oysters is disruptions to polar lobe formation. The polar lobe is a unique structure found in embryogenesis in some molluscs that possesses very important cell differentiation information. They are formed in at least the first two mitotic divisions. Observations in this study show that if shock(s) are applied at the first mitotic division, the proportion of eggs dividing equally at next cell cycle will increase relative to the eggs

in control. However, no evidence is available on to what extent an embryo could tolerance this kind of disruption to the polar lobe.

Guo *et al.* (1994) hypothesized that the poor viability of induced tetraploids was caused by a cell number deficiency due to the cleavage of normal eggs by large tetraploid nuclei, resulting in a reduction in cell number at a developmental stage. Thus further morphogenesis may stop, because most molluscs follow a "mosaic" type of development where a cell's fate is programmed by the number of divisions and distribution of morphogenic determinants (Gilbert 1988, cited in Guo *et al.* 1994). The small number of tetraploids produced from normal eggs survived metamorphosis in mussels, *Mytilus galloprovincialis* (Scarpa *et al.* 1993) and the Pacific oysters, *C. gigas* (the present study) suggests that some embryos or species can tolerate a cell number deficiency well and develop into normal animals.

BENEFITS

If this report was completed as scheduled in 1998 the benefits of this project to the oyster industry would have been minimal, as the performance of triploid oysters produced by the chemical method were of very poor quality on the trial leases in South Australia. The shell growth rate of triploids was comparable to their diploid siblings, however their meat was very watery and unmarketable. Grower demand for triploid oysters had consequently fallen considerably. As a result, plans for continued research into the production of tetraploid broodstock were abandoned.

However, recent results from triploid stock produced by crossing tetraploids with diploids showed that on some leases natural triploids (interploid crosses) grew faster than their diploid siblings and their meat remained of high quality after diploids spawned during summer (Nell 2002). In the Southern Hemisphere, summer includes Christmas and New Year and is the time of the year when consumer demand for oysters is at its highest. These results have inspired some growers to farm triploids in Australia again. If the tetraploid spat produced in this project could retain their ploidy status at mature ages and significant numbers of males and females were found, the breeding population could then be established by direct crossing of tetraploid broodstock. If the tetraploids were used for commercial production of natural triploids in Australia, the benefits to the oyster industry would be significant.

FURTHER DEVELOPMENT

Tetraploid Pacific oyster spat have been induced in this study using the combined chemical method, however, at this stage no information is available on their performance at maturity. Research on other bivalve species indicates that the ploidy levels in tetraploids induced directly from eggs produced by diploid females are not stable and tetraploids were not found when sampled at a later date (Yang *et al.* 2000). To date, no tetraploid breeding populations have been established from the tetraploids produced directly from diploid females.

To further improve the techniques developed in this study, non-destructive ploidy analysis techniques for spat is needed so that the development of individual tetraploid spat can be monitored and the reversal of tetraploids to mosaics or lower levels of euploids, if any, can be confirmed. If the rate of reversal were too high, the establishment of a breeding line from the tetraploids induced from the eggs of diploid females would be very difficult.

The chemical 2-mercaptoethanol was first used in ploidy manipulation in this study. Detailed studies on chromosome and centrosome behaviours and molecular changes in cell membranes of eggs treated with CB and 2-mercaptoethanol will be needed to determine the mechanism involved in improving tetraploid induction.

The results from experiments indicate that the tolerance of oysters to 2-mercaptoethanol may vary between different stocks. The survival rate of eggs treated with the combined chemicals varied, with high survival in some stocks and total mortality at day 5 in others. This may be a result of differences in their genetics or physiological conditions. The broodstock used in this study were mainly purchased from fish markets on the same day the oysters were delivered.

PLANNED OUTCOMES

The expected outcomes from this study were the production of tetraploid oyster embryos, larvae and spat; the ongrowing of tetraploid spat to adulthood to establish broodstock and the production of 100% commercial triploid stock, by crossing diploid and tetraploid broodstock.

The planned outcomes of this project were not achieved, although tetraploid spats were induced. Plans to continue research into the production of tetraploid broodstock were abandoned due to a fall in grower demand for triploid oysters as a result of poor growth rates and meat quality of chemically produced triploid oysters on commercial leases. In addition to this, tetraploids did not

survive beyond metamorphosis from experiments conduced in the first year. To achieve the planned outcomes, the project would need to be extended for one to one and a half years, as normally two years are required for diploid oysters to grow to a size that could be used as broodstock for commercial production.

CONCLUSION

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

1. Tetraploid induction by electrofusion

   This method was successful in terms of fusing the two blastomeres in 2-cell embryos. However, re-division of these embryos was abnormal, resulting in 3 or 4-cells after next cleavage.

2. Tetraploid induction by suppression of mitotic I division.

   Tetraploid larvae were induced by most methods tried in this study, including physical (thermal, heat + caffeine, and hydraulic pressure) and chemical (CB and 6-DMAP) treatments. In general, the physical methods were more effective than the chemical treatments, resulting in a higher percentage of tetraploid larvae. No tetraploids, however, survived beyond metamorphosis.

3. Tetraploid induction by suppression of both polar body formations.

   Experiments where 1 mg/L of CB was used were the most effective, resulting in 35 % tetraploid Pacific oyster larvae. Larvae produced in other experiments were mainly pentaploid. No larvae survived beyond the metamorphosis stage in this study.

4. Tetraploid induction by suppression of first polar body formation.

   This method was tried numerous times in the present studies. However, the resultant larvae were mainly aneuploids, with no tetraploid larvae developing into spats.

5. The combined chemicals method

This method was developed in this project. Tetraploid oyster spat were produced in this study. This provides the first evidence that tetraploid Pacific oysters produced with eggs of diploid females can survive beyond the metamorphosis stage and develop into spat. In addition to this, the percentage of tetraploids produced with combined chemicals (>50 %) was substantially higher than the results from CB treatments only (about 30%; Guo *et al.* 1992a; Yang *et al.* 2000; and present study), which suggests that this method could also increase the numbers of eggs going through "separated bipolar segregation" after the first meiotic division is inhibited. This may be the reason for the production of tetraploid oyster spats in this study.

SUMMARY

It should be noted that whilst successful tetraploid inductions from eggs produced by diploid females, have been reported in bivalves for more than 10 years, to date no breeding lines have been established from these tetraploids. This maybe due to: 1) the published methods are difficult to reproduce, therefore the number of tetraploids produced is not large enough to establish a breeding population; 2) the tetraploids produced reverse to mosaics or lower ploidy levels; or 3) a combination of both 1 and 2. Therefore, more research is needed to further improve these tetraploid induction techniques, especially for those bivalve species in which tetraploids cannot be produced using eggs of triploid females.

REFERENCES

Ahmed, M., 1973. Cytogenetics of oysters. Cytologia, 38: 337-347.

Allen Jr., S.K., 1987. Genetic manipulations: critical review of methods and performances in shellfish. In: Selection, Hybridization and Genetic Engineering in Aquaculture. Proceedings of a World Symposium. Tiews, K. (ed.). Berlin: Schriften der Bundesforschungsanstalt fur Fischerei Hamburg Band 18/19.

Allen Jr., S.K., and Downing, S.L., 1990. Performance of triploid Pacific oysters, *Crassostrea gigas*. Gametogenesis. Can Jour. Fish. Aquat. Sci., 47:1213-1222.

Allen Jr., S.K., Downing, S.L., Chaiton, J.C. and Beattie, J.H., 1986. Chemically and pressure induced triploidy in the Pacific oyster, *Crassostrea gigas*. Aquaculture, 57: 359-360 (abstract).

Allen Jr., S.K., Downing, S.L. and Chew, K.K., 1989. Hatchery Manual for Producing Triploid Oysters. Seattle. WA: University of Washington Press.

Allen Jr., S.K., Guo, X., Burreson, G., and Mann, R., 1996. Heteroploid mosaics and reversion among triploid oysters, *Crassostrea gigas*. Fact or artefact. J. Shellfish Res., 15: 514 (Abstract only).

Allen Jr., S.K., Shpigel, M., Utting, S. and Spencer, B., 1994. Incidental production of tetraploid Manila clams, *Tapes philippinarum* (Adams and Reeve). Aquaculture, 128: 13-19.

Allen Jr., S.K., and Stanley, J.G., 1979. Polyploid mosaics induced by cytochalasin B in landlocked atlantic salmon *Salmo salar*. Trans. Am. Fish. Soc., 108: 462-466.

Allen Jr., S.K., and Stanley, J.G., 1983. Ploidy of the hybrid grass carp X bighead carp determined by flow cytometry. Trans. Am. Fish. Soc., 112: 431-435.

Arai, K.F., Naito, F. and Fujino, K., 1986. Triploidization of the Pacific abalone with temperature and pressure treatment. Bull. Japan Soc. Sci. Fish., 52: 417-422.

Beaumont, A. and Fairbrother, J.E., 1991. Ploidy manipulations in molluscan shellfish: a review. J. Shellfish Res., 10(1): 1-18.

Cadoret, J.P., 1992. Electric field-induced polyploidy in mollusc embryos. Aquaculture, 106: 127-139.

Chaiton, J.A and Allen Jr., S.K., 1985. Early detection of triploidy in the larvae of Pacific oysters, *Crassostrea gigas*, by flow cytometry. Aquaculture, 48:35-43.

Chourrout, D., 1984. Pressure-induced retention of second polar body and suppression of first cleavage in rainbow trout: production of all-triploids, all-tetraploids, heterozygous and homozygous diploid gynogenetics. Aquaculture, 36: 111-126.

Coon, S.L., Bonard, D.B. and Weiner, R.M., 1986. Chemical production of cultchless spat using epinephrine and norepinephrine. Aquaculture, 58: 255-262.

Downing, S.L. and Allen Jr., S.K., 1987. Induction triploidy in the Pacific oyster, *Crassostrea gigas*: Optimal treatments with cytochalasin B depend on temperature. Aquaculture, 61: 1-15.

Desrosiers, R. R., Gerard, A. and Peignon, J.M., 1993. A noval method to produce triploids in bivalve mollusks by the use of 6-dimethylaminopurine. J. Exp. Mar. Biol. Ecol., 170: 29-43.

Eudeline, B., Allen Jr., S.K. and Guo, X., 2000. Optimization of tetraploid induction in the Pacific oysters, *Crassostrea gigas*, using the first polar body as a natural indicator. Aquaculture, 187: 73-84.

Fischberg, M., 1958. Experimental tetraploidy in newts. J. Embryol. Exp. Morphol., 6: 393-402.

Gerard, A., Naciri, Y., Peignon, J.M., Ledu, C. and Phelipot, P., 1994. Optimization of triploid induction by the use of 6-DMAP for the oyster *Crassostrea gigas* (Thunberg). Aquaculture and Fisheries Management, 25: 709-719.

Guo, X., 1999. Superior growth as a general feature of triploid shellfish: evidence and possible causes. J. Shellfish Res., 18: 266-267.

Guo, X., and Allen Jr., S.K., 1994. Viable tetraploids in the Pacific oyster (*Crassostrea gigas* Thunberg) produced by inhibiting polar body I in eggs from triploids. Mol. Mar. Biol. Biotechnol., 31: 42-50.

Guo, X., DeBrosse, G. A. and Allen Jr., S.K., 1996. All-triploid Pacific oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. Aquaculture, 142: 149-161.

Guo, X., Cooper, K., Hershberger, W.K. and Chew, K.K., 1992a. Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster, *Crassostrea gigas*: I. Ploidy of resultant embryos. Biol. Bull., 183: 381-386.

Guo, X., Cooper, K., Hershberger, W.K. and Chew, K.K., 1994. Tetraploid induction with mitosis I inhibition and cell fusion in the Pacific oyster (*Crassostrea gigas* Thunberg). J. Shellfish Res., 13: 193-198.

Guo, X., Hershberger, W.K., Cooper, K. and Chew, K.K., 1992b. Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster, *Crassostrea gigas*: II. Segregation of chromosomes. Biol. Bull. 183: 387-393.

Guo, X., Hershberger, W.K., Cooper, K. and Chew, K.K., 1993. Artificial gynogenesis with ultraviolet light-irradiated sperm in the Pacific oyster, *Crassostrea gigas*. I. Induction and survival. Aquaculture, 113: 201-214.

Guo, X., Wang, J., Landau, B.J., Li, L., DeBrosse, G.A. and Krista, K.D., 2002. The successful production of tetraploid eastern oyster, *Crassostrea virginica* Gmelin. Program and Abstract of the 94th Annual Meeting of the National Shellfishery Association, April 14-19, Mystic, CT, pp. 22-23 (Abstract only).

He, M., Lin, Y., Shen, Q., Hu, J. and Jiang, W., 2000. Production of tetraploid pearl oyster (*Pinctada martensii* Dunker) by inhibiting the first polar body in eggs from triploids. J. Shellfish Res., 19: 147-151.

Komaru, A., Scarpa, J. and Wada, K.T., 1995. Sperm production in tetraploid mussels, *Mytilus galloprovincialis*. Aquaculture '95. California USA. (abstract only).

Komaru, A. and Wada, K.T., 1989. Gametogenesis and growth of induced triploid scallops, *Chlamys nobilis*. Nippon Suisan Gakkaishi, 55: 447-452.

Li, X., 1996. The effects of centrosomes on chromosome manipulation in commercial marine invertebrates. Oceanologia et Limnologia, 27(3):207-214.

Li, X. and Chen, T.J., 1996. Microtubule-organizing centres and cell division. Life Sciences, 14: 214-219.

Longo, F.J., Mathews, L. and Hedgecock, D., 1993. Morphogenesis of maternal and paternal genomes in fertilized oyster eggs (*Crassostrea gigas*): Effects of cytochalasin B at different periods during meiotic maturation. Biol. Bull., 185: 197-214.

Myers, J.M., Iwamoto, R.N. and Hershberger, W.K., 1986. Tetraploid induction in salmonids. J. World Aqua. Soc., 17: 1-7.

Nell, J.A., 2002. Farming triploid oysters. Aquaculture, 210: 69-88.

Nell, J.A., Hand, R.E., Goard, L.J., McAdam, S.P. and Maguire, G.B., 1996. Studies on triploid oysters in Australia. V. Evaluation of cytochalasin B and 6-dimethylaminopurine for triploidy induction in Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley). Aquaculture Res., 27: 101-110.

Peruzzi, S. and Guo, X., 2002. Tetraploid induction by meiosis inhibition with cytochalashin B in the dwarf surfclam, *Mulinia latetralis* Say: effects of temperature. J. Shellfish Res., 21: 677-684.

Quillet, E. and Panelay, P.J., 1986. Triploidy induction thermal shocks in the Pacific oyster, *Crassostrea gigas*. Aquaculture, 57: 271-279.

Scarpa, J., Toro, J. and Wada, K., 1994. Direct comparison of six methods to induce triploidy in bivalves. Aquaculture, 119: 119-133.

Scarpa, J., Wada, K. and Komaru, A., 1993. Induction of tetraploidy in mussels by suppression of polar body formation. Nippon Suisan Gakkaishi, 59: 2017-2023.

Shen, Y.P., Zhang, X.Y., He, H.P. and Ma, L.J., 1993. Triploidy induction by hydrostatic pressure in the pearl oyster, *Pinctada martensii* Dunker, Aquaculture, 110: 221-227.

Stephens, L.B. and Downing, S.L., 1988. Inhibiting first polar body formation in *Crassostrea gigas* produces tetraploids, not meiotic triploids. J. Shellfish Res., 7: 550-551 (Abstract only).

Thorgaard, G.H., Jazwin, M.E. and Stier, A.R., 1981. Polyploidy induced by heat shock in rainbow trout. Trans. Am. Fish. Soc., 110: 546-550.

Varadaraj, K. and Pandian, T.J., 1988. Induction of triploids in *Oreochromis mossambicus* by thermal, hydrostatic pressure and chemical shocks. Pro. Aquaculture Int. Congr. and Expo., 531-535.

Yamamoto, S. and Sugawara, Y., 1988. Induced triploidy in the mussel, *Mytilus edulis*, by temperature shock. Aquaculture, 72: 21-29.

Yamamoto, S., Sugawara Y. and Nomura, T., 1990. Chemical and thermal control of triploid production in Pacific oysters and mussels, with regard to controlling meiotic maturation. Adv. Invert. Reprod., 5: 455–460.

Yang, H., Zhang, F. and Guo, X., 2000. Triploid and tetraploid Zhikong scallop, *Chlamys farreri* Jones et Preston, produced by inhibiting polar body I. Marine Biotech., 2: 466-475.

Zimmermann, U., 1987. Electrical breakdown, electropermealization and electrofusion. Rev. Physiol. Biochem. Pharmacol., 105: 175-256.

APPENDIX 1: Intellectual Property

The combined chemicals method developed in this project has substantially improved the tetraploid levels in the resultant embryos and produced tetraploid oyster spats (analysed by flow cytometry) in this study. This technique has the potential to improve chromosome manipulation techniques that target the first meiotic division.

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