Tetraploidy induction in Sydney rock oysters

by

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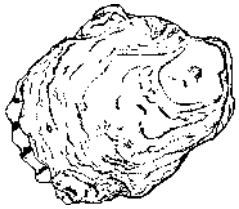

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Project D.4.2

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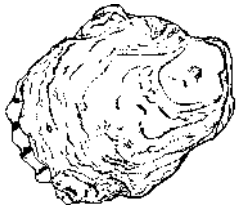

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AQUACULTURE CRC
FINAL PROJECT REPORT
1 JULY 1994 - 30 JUNE 1998

PROJECT DETAILS

Project Number and Title

D.4.2 Tetraploidy induction in Sydney rock oysters

Project Leader

John A Nell, NSW Fisheries, Taylors Beach, NSW

Research Institutions actively involved in the CRC project

1. NSW Fisheries
2. Rutgers University, Port Norris, NJ
3. Flinders University, Adelaide, SA
4. FuCell Pty Ltd, Department of Biophysics, University of New South Wales, Kensington, NSW

Industry organisations actively involved in the CRC project

1. Oyster Farmer's Association of NSW Ltd
2. NSW Farmers' Association (Oyster Section)

Main aims of the project

1. To induce tetraploidy in Sydney rock oyster larvae.
2. To rear tetraploid Sydney rock oyster larvae through metamorphosis to spat.
3. To establish of one or more breeding lines of tetraploid Sydney rock oysters. These tetraploid (4n) oysters were to be crossed with diploid (2n) oysters from an improved breeding line for the production of all triploid (3n) oysters. These triploid oysters would need to be produced by a commercial hatchery for sale to oyster farmers.

ACHIEVEMENTS OF THE PROJECT

Principal achievements

Method 1 - *Blocking of extrusion of both polar body 1 and 2 in eggs from diploids fertilised by sperm from diploids*

An attempt in March 1995 to block extrusion of polar bodies 1 and 2 using cytochalasin B resulted in 21% tetraploids on day 0. However, early larval mortality was high 98% and no tetraploids survived to day 7.

Method 2 - *Inhibition of polar body 1 in eggs from triploids fertilised by sperm from diploids*
This method was developed by Drs Ximing Guo and Standish Allan, Rutgers University, Port Norris, NJ.

In January 1996, a tetraploid induction produced day 0 tetraploidy levels ranging from 4-8% (as measured by direct chromosome count) but no tetraploids were found when spat were four months old. A second attempt in February 1997 produced day 0 tetraploidy levels of 13 and 10% (as measured by direct chromosome count) for tanks 1 and 2 respectively. By day 6 they had dropped to 7 and 5% (as measured by flow cytometry) respectively. No tetraploids were found when spat were four months old.

Method 3 - *Treatment of eggs from diploids fertilised by sperm from diploids*
This method was developed by, Drs Li Xiaoxu and Jon Havenhand, Flinders University, Adelaide, SA. An April 1997 tetraploid induction produced a day 0 tetraploidy level of 8% (as measured by direct chromosome count) but by day 13 no tetraploids had survived (as measured by flow cytometry). A series of four replicated experiments were conducted to optimise tetraploidy induction conditions from November 1997 to January 1998. Unfortunately induction success was not improved by any of the treatments tested. Experimental details are subject to a confidentiality agreement between NSW Fisheries and Flinders University.

A series of 12 large scale induction attempts were carried out over February/March 1998. Day 0 tetraploidy levels ranged from 0 to 5% (as measured by direct chromosome count), unfortunately no tetraploids were found by day 5 (as measured by flow cytometry).

Method 4 - *Electrofusion of individual two-cell embryos*
Method developed by Professor Hans Coster (FuCell Pty Ltd, Department of Biophysics, University of New South Wales, Kensington, NSW). Between 1996/97, using an electric pulse, individual two cell embryos were fused. The experimental cell fusion technique produced small numbers of fusates per day (20-40 estimated), but the maximum production possible has yet to be determined. With the survival of Sydney rock oysters from fertilisation to 3 months old spat calculated as 5-10%, more than 400 fusates would be required to produce 20 surviving spat. This is beyond the capacity of the technique at present.

Overall progress towards achieving main aim of project

Although all four methods were successful in producing tetraploids immediately after treatment (as measured by direct chromosome count) none of these could be reared to viable oyster spat, so the main objective of the project was not achieved.

PUBLICATIONS CONTAINING SIGNIFICANT MATERIAL FROM CRC PROJECT

Papers, books or chapters in preparation
Nil

Papers, books or chapters accepted for publication
Nil

Conference presentations made
Nil

Provisional patents filed
Nil

Patents completed
Nil

APPLICATION OF RESEARCH WITHIN SCOPE OF CRC PROJECT

Details of publications, manuals and other documents transferring know-how to industry
Nil

Transfers of animals, materials, techniques, software, or other forms of know-how to industry/end users
Presentation at 'Oyster Industry Field Day 1997, at the Port Stephens Research Centre, Taylors Beach, NSW on 11 September 1997.

Workshops, seminars, training courses
Nil

EDUCATIONAL OUTCOMES RELATING DIRECTLY TO CRC ACTIVITIES

PhD's awarded to students on CRC project
Nil

Formal postgraduate qualifications, other than PhD's, awarded to students on CRC project
Nil

SCIENTIFIC REPORT

Tetraploid Induction in Sydney rock oyster *Saccostrea commercialis*

Abstract

Four tetraploid induction techniques were tested on Sydney rock oysters *Saccostrea commercialis*. The first one was by blocking of both polar bodies 1 and 2 in eggs from diploids fertilised by sperm from diploids and the second one by blocking of polar body 1 in eggs from triploids fertilised by sperm from diploids. Both methods successfully produced tetraploid larvae immediately after treatment but few, if any, tetraploids survived through to metamorphosis (low levels were detected using flow cytometry). No tetraploids were detected in spat from either method when analysed by direct chromosome count. A third method, which is subject to a confidentiality agreement with Flinders University, Adelaide, was tested. This method used treatment of eggs from diploids fertilised by sperm from diploids, which again produced tetraploid larvae at day 0, but none of these survived to day 5.

A fourth method using individual electrofusion of two cell embryos produced several fusates which survived for 1 day or a little more. The experimental cell fusion technique produced small numbers of fusates per day (20-40 estimated), but the maximum production possible has yet to be determined. With the survival of Sydney rock oysters from fertilisation to 3 months old spat calculated as 5-10%, more than 400 fusates would be required to produce 20 surviving spat. This is beyond the capacity of the technique at present.

1. Introduction

The aims were to induce tetraploidy in Sydney rock oyster larvae and to rear tetraploid Sydney rock oyster larvae through metamorphosis to spat to establish one or more breeding lines of tetraploid Sydney rock oysters. These (4n) tetraploid oysters were to be crossed with diploid (2n) oysters from an improved breeding line for the production of all triploid (3n) oysters. These triploid oysters would need to be produced by a commercial hatchery for sale to oyster farmers.

Tetraploidy in bivalves has been successfully induced by blocking both polar bodies 1 and 2 in mussel eggs *Mytilus galloprovincialis* (Scarpa et al., 1993) and by blocking polar body 1 in eggs from triploids fertilised by sperm from diploids in Pacific oysters *Crassostrea gigas* (Guo and Allen, 1994). Another method developed by, Drs Li Xiaoxu and Jon Havenhand, Flinders University, Adelaide, SA, with potential for tetraploidy induction in oysters involves treatment (subject to a confidentiality agreement) of eggs from diploids fertilised by sperm from diploids. A fourth method, electrofusion of two cell embryos also showed promise. All four methods were tested for induction of tetraploidy in the Sydney rock oyster.

2. Materials and methods

Method 1 - Blocking of both extrusion of polar body 1 and 2 in eggs from diploids fertilised by sperm from diploids - March 1995

Broodstock

In March 1995, a batch of pooled eggs (598 million) from 71 females was fertilised by sperm from 30 males. These eggs were then divided among six 20 L aquaria and tetraploidy induced. Eggs and sperm were pooled separately before fertilisation (Nell et al., 1996) within 1.5 h post-spawning. Preliminary research has indicated the need to use large numbers of eggs because of very high mortalities associated with the treatment.

Tetraploidy induction

Pooled eggs were treated from 1 min post-fertilisation with 1 mg cytochalasin B (CB)/L in 0.1% dimethylsulfoxide (DMSO) for 40 min at 25±1^{o}C (Scarpa et al., 1993), then rinsed for 15 min in 0.1% DMSO before being stocked directly into three 20 000 L tanks (two 20 L aquaria or 199 million eggs per tank).

Larval and spat rearing

Larvae were reared and fed as described by Nell et al., 1996. The first water change was on day 2 and surviving larvae from the 3 tanks were re-stocked into one 20 000 L tank. After day 2 water was changed at 2-4 day intervals. Larvae were put to set as ‘single seed’ on ground scallop shell in downwellers from days 19-23. Spat were reared in upwellers in an outdoor nursery system. In November 1995 and May 1996 spat were graded, analysed for ploidy level and the largest size grade kept each time.

Method 2 - Blocking of polar body 1 in eggs from triploids fertilised by sperm from diploids

January 1996

Broodstock

The ploidy status of 690 individual oysters from a triploid batch produced in March 1995 was determined by flow cytometry (Allen et al., 1994) and 624 oysters were within the triploid range; however, only those oysters that were definite triploids (600 oysters) were used in the induction trial. These oysters were sexed and egg quality was checked by gonad biopsy (Nell et al., 1996). Only 101 females with eggs in reasonable condition were found, approximately 15% of the total oysters tested. In January 1996, these 101 triploid females were strip spawned; however, only 34 females were used due to the poor quality and low number of eggs from the remainder. Male broodstock was taken from a batch of diploid oysters produced in March 1995 from the same fertilised eggs as those used for the triploid batch. Sperm from 11 males was pooled and used to fertilise the triploid eggs within 1.5 h of the start of strip spawning.

Tetraploidy induction

The 5.8 million fertilised eggs were divided between two 2 L beakers and treated from 5 min post-fertilisation with 1 mg CB/L in 0.1% DMSO for 20 or 30 min at 25±1^{o}C (Guo and Allen, 1994). Treated eggs were then rinsed for 15 min in 0.1% DMSO before being stocked directly into one 10 000 L tank for each treatment.

Larval and spat rearing

Larvae were reared and fed as described by Nell et al. (1996). The first water change was on day 3 and surviving larvae were re-stocked into one 1 000 L tank for each treatment. Subsequent water changes were at 2-3 day intervals. From day 21-32, larvae >300 µm were screened out and treated with 10^{-4} M epinephrine bitartrate for 45 min to induce metamorphosis (Beiras and Widdows, 1995) before being transferred to a downweller. Larvae in the downwellers that had not metamorphosed after 2 or 3 days were retreated with epinephrine. Spat were reared in upwellers in an indoor nursery system for approximately 6 weeks then transferred to an outdoor upweller system.

February 1997

Broodstock

Triploids (89% triploidy) were produced from 48 females and 6 males in a mass spawning of oysters in February 1994. In January/February 1997, 358 oysters were sexed by gonad biopsy and 53 females were found, 20 of which were shown to be triploids after flow cytometry on individual gill tissue samples. Broodstock were held at 28^{o}C for 1 - 2 weeks before strip spawning.

Spawning

In February 1997, triploid females and diploid males were strip spawned. Two of the 19 strip-spawned females, produced sperm as well as eggs, so their eggs were discarded. Eggs were not washed to prevent risk of losing small eggs that had not rounded up before fertilisation and CB application.

Treatment

Pooled eggs (63x10^{6}) were divided between 2 (10 L) aquaria and treated from 5 min post-fertilisation with 1 mg cytochalasin B (CB)/L in 0.1% dimethylsulfoxide (DMSO) for 30 min in 20 L at 25±1^{o}C. Then they were rinsed for 15 min in 0.1% DMSO before being stocked directly into 2 (20 000 L) tanks. At day 2, surviving larvae were re-stocked in a 1 000 L tank.

Larvae rearing and spat settlement

Larvae were stocked at low densities (<3/mL) and set on scallop shell (no epinephrine or other chemical was used).

Method 3 - Treatment of eggs from diploids fertilised by sperm from diploids

This method was developed by Drs Li Xiaoxu and Jon Havenhand, Flinders University, Adelaide, SA. In April 1997, 33 diploid female oysters spawned naturally and 12 diploid male oysters were strip spawned and 100 million eggs were treated. Larvae were reared in a 20 000 L tank. A series of four replicated experiments were conducted to optimise tetraploidy induction conditions from November 1997 to January 1998. Larvae were reared in 8 L aquaria for 5 days. Experimental details are subject to a confidentiality agreement between NSW Fisheries and Flinders University.

A series of 12 large scale induction attempts were carried out over February/March 1998. Treated eggs were stocked at 25/mL in a 2 000 L tank (seawater salinity 35λ and temperature 25±1°C) and larvae were reared for 5 days.

Method 4 - Individual electrofusion of individual two-cell embryos - November 1996 - November 1997

Method developed by Professor Hans Coster (FuCell Pty Ltd, Department of Biophysics, University of New South Wales, Kensington, NSW). Oysters were strip spawned and eggs fertilised. Two-cell embryos were transferred into a 1 000 mOsm (equivalent to seawater osmolarity) sucrose solution and fused one by one with an electric pulse. After fusion embryos, were transferred and larvae reared in 1 L conical flasks containing 500 mL seawater and 1g/1000 L ethylenediaminetetra-acetic acid (EDTA) in an orbital shaker-incubator at 25°C.

Ploidy analysis

Larvae

Triploidy percentages in day 0 larvae and large spat (>10 mm) were determined by direct chromosome count. For larvae the method of Allen et al. (1989), modified by Nell et al. (1996) was used, the procedures used for chromosome counts on older spat are described below. Direct chromosome counts are more precise than flow cytometry for the determination of the ploidy status of oysters. In the case of larvae direct chromosome counts can only be done readily in the trochophore stage (Gérard et al., 1991; Nell et al., 1996); triploidy percentages in older larvae and small spat were therefore determined by flow cytometry (Chaiton and Allen, 1985).

Spat - direct chromosome counts

The technique of preparing chromosome spreads was adapted from Allen et al. (1989). Spat were placed in 0.025% colchicine (from a stock solution of 1% in distilled water, kept frozen) in aerated, 50% seawater at 20-25^{0}C for 16-24 h. Spat were encouraged to filter by adding algae.

Spat were then removed from colchicine. A piece or all of the gill was dissected out of each and macerated with a scalpel. In very small spat the whole body was used. Gill tissue was placed into individual 10 mL centrifuge tubes in approximately 4 mL of 0.6% sodium citrate in distilled water for 15 min at ambient temperature. Sodium

citrate was pipetted off and replaced with fresh solution for a further 15 min (alternatively 0.9% sodium citrate may be used in two 30 min exposures; however, the 0.6% solution appeared to give superior spreads).

Approximately 0.5 mL of Carnoys fixative (3:1 absolute methanol: glacial acetic acid) was added to gill tissue in sodium citrate solution, mixed and left for 2 min. The solution was then pipetted off.

Full strength fixative (made fresh each time due to the tendency for glacial acetic acid to absorb water) was added at 10x tissue volume for 12 h changing the solution every 2 h. At this stage tissue can be stored for up to 6 months at 5ºC in Carnoys fixative.

Tissue was removed from Carnoys fixative and placed in individual watch glasses, dabbed dry with paper towel and replaced with a few drops of 50% glacial acetic acid (in distilled water). Each sample was minced in glacial acetic acid for 3-4 min then the suspension was pipetted off and dropped onto a warmed slide (45^{o}C) from a height of approximately 10 cm. This helped to spread the cells on the slide. Drops were aspirated off the slide repeatedly with a pipette until there were several rings of cells on the slide.

Slides were left to air dry then rinsed in methanol for 10-15 min to remove any acetic acid residue. 10% Giesma stain (in pH 6.8 phosphate buffer) was applied for 10-15 minutes to stain the chromosomes. Slides were then rinsed with tap water followed by a final rinse with distilled water. When dry, slides were examined under 40x magnification for chromosome number. If the quality of the chromosome spread was poor and chromosomes could not be accurately counted, cover-slips were permanently fixed to the slides and they were examined under 100x oil immersion.

3. Results

Method 1 - Blocking of both polar bodies in eggs from diploids fertilised by sperm from diploids - March 1995

On day 0 there were approximately 21% tetraploids (Table 1), however, early larval mortality was very high (Table 1) and few tetraploids (Table 2) survived to day 7 (as measured by flow cytometry detection limit is around 3%). Only 2 million spat were produced from the combined 3 tanks (0.3% survival). There was also very high mortality in spat from May-November 1995. Mortality was very high (>90%) in the smallest (1.0 -1.4 mm) size grades, but much lower (7-50%) in the largest (1.4-4.2 mm) size grades. In November 1995 ploidy analysis by flow cytometry indicated that there could be around 4% tetraploids in the two largest size grades (Table 3). However direct chromosome count of the largest spat in May 1996 found no tetraploids (Table 4).

Method 2 - Blocking of polar body 1 in eggs from triploids fertilised by sperm from diploids

January 1996

Only 5.8 million eggs were produced from 34 ripe triploid females ie an average of 170 000 eggs each. Early mortality was high with only 13% and 14% of larvae surviving to day 6 for the 20 min and 30 min treatments respectively (Table 5). Tetraploidy levels for the 20 min duration were 4% at day 0 and day 6; tetraploid levels were slightly higher for the 30 min treatment with 8 and 7% on day 0 and day 6 respectively (Table 6). At metamorphosis only 1 500 and 9 400 spat remained for the 20 and 30 min treatments respectively. Spat were combined into one batch several weeks after metamorphosis. No tetraploids were found amongst the spat by direct chromosome count in June 1996 (Table 7).

February 1997

Details of larval survival are shown in Table 8. Day 0 tetraploidy levels were 13 and 10% (as measured by direct chromosome count) for tanks 1 and 2 respectively. By day 6 they had dropped to 7 and 5% (as measured by flow cytometry) respectively (Table 9). No tetraploids were found when spat were four months old (Table 10).

Method 3 - Treatment of eggs from diploids fertilised by sperm from diploids

Day 0 tetraploidy level was 8% (as measured by direct chromosome count) but by day 13 no tetraploids had survived (as measured by flow cytometry). For the four replicated experiments conducted to optimise tetraploidy induction conditions, induction success was not improved by any of the treatments. Tetraploid levels in spat were <3% error level.

Egg fertilisation and survival date for the large scale induction attempts, (Table 11), day 0 tetraploidy levels (Table 12) ranged from 0 to 5% (as measured by direct chromosome count), unfortunately by day 5 tetraploidy level was <3% error level (Table 12) (as measured by flow cytometry).

Method 4 - Individual electrofusion of two-cell embryos

Several fusates have been produced, that survived for ≥1 day. The cell fusion technique can only produce very small numbers of larvae (eg 20 - 40/day). The actual daily production limits still need to be tested. Survival from fertilised eggs to 3 months old spat in Sydney rock oysters is around 5-10% for successful batches. To rear 20 spat, >400 fusates would need to be produced. This is currently beyond the capacity of this technique.

4. Discussion

The only report of viable tetraploid oysters is that of tetraploid Pacific oysters *Crassotrea gigas* produced by inhibiting polar body 1 in eggs from triploids fertilised by sperm from diploids (Guo and Allen, 1994; Guo et al., 1996). These tetraploids were formed through united bipolar chromosome segregation at meiosis I (Que et al.,

1997). This was most likely the reason, that the tetraploid Pacific oyster larvae produced by (Guo and Allen, 1994; Guo et al., 1996) survived through metamorphosis. Chromosome segregation was not investigated in this study, tripolar segregation had been observed. This may have been one of the causes of mortality of the tetraploid Sydney rock oyster larvae in this study. Of the four methods of tetraploidy induction attempted, three were thoroughly investigated in this study. These methods were: blocking of both polar bodies in eggs from diploids fertilised by sperm from diploids, blocking of polar body 1 in eggs from triploids fertilised by sperm from diploids, and treatment of eggs from diploids fertilised by sperm from diploids. All produced tetraploid larvae but no tetraploid spat. Unless these methods can be modified, further attempts at tetraploidy in Sydney rock oysters using these three techniques cannot be justified. However, the fourth method, electrofusion of two cell embryos (one at a time) has merit and the process needs further development to allow the production of hundreds of fusates in a single working session.

Acknowledgments

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Table 1

Method 1 - The fertilisation, survival and chromosome number, determined by direct chromosome counts, of *Saccostrea commercialis* larvae at day 0-2, sampled from each of the three tanks (20 000 L) in March 1995.

Tank	Fertilisation (%)	Day 2 survival (%)	Day 0 chromosome number[1] (% larvae)							
			<18	18-22	23-27	28-32	33-37	38-42	43-47	48-52
1	97	2.9	2	32	15	30	2	18	0	2
2	96	3.3	0	28	8	32	5	23	2	2
3	97	1.5	0	20	10	43	3	22	0	2

[1]n=60 larvae; determined by direct chromosome count.

Table 2

Method 1 - Larval rearing results from March-April 1995.

Day	Number ($x10^6$)	Chromosome number (% larvae)[1]							
		<18	18-22	23-27	28-32	33-37	38-42	43-47	48-52
0	598	1	27	11	35	3	21	1	2
2	15.2	-	-	-	-	-	-	-	-
7	11.2	0	11	9	59	17	3	0	0
15	6.5	0	22	18	50	6	2	0	0
19-23	4.8 put to set[2]	-	-	-	-	-	-	-	-

[1]n=3 samples of larvae, determined by flow cytometry for days 7 and 15.
[2]The proportion of larvae that set was 42%.

Table 3

Method 1 - Spat rearing results from May - November 1995.

Month	Grade[2] (mm)	Number (x1000)	Chromosome number[1] (% spat)						
			18-22	23-27	28-32	33-37	38-42	43-47	48-52
May '95	0.7-1.8	2014	-	-	-	-	-	-	-
June '95		800	-	-	-	-	-	-	-
Sept '95		300	-	-	-	-	-	-	-
Nov '95	>8.5	30[3]	4[4]	2	54	36	4	0	0
	7.1-8.5	69[3]	8	20[4]	50	18	4	0	0
	4.2-7.1	189	16[4]	14[4]	62	8	0	0	0
	2.0-4.2	46	8	24	66	2	0	0	0

[1]n=50 individual spat for each grade; determined by flow cytometry.
[2]Diagonal mesh size of screens used for grading.
[3]These oysters were selected for on-growing, all others were disposed of.
[4]Includes mosaic (mixture of cell chromosome numbers) oysters.

Table 4

Method 1 - The total number of Sydney rock oyster *Saccostrea commercialis* spat in each grade and the chromosome number of those in the two largest grades in May 1996.

Grade[2] (mm)	Number of spat	Chromosome number[1] (% spat)							
		18-22	23-27	28-32	33-37	38-42	43-47	48-52	mosaic
>25.5	1420[3]	56	3	25	-	-	-	-	17[4]
22.6-25.5	3150[3]	44	-	31	-	-	-	-	25[5]
19.8-22.6	5770	-	-	-	-	-	-	-	-
<19.8	23810	-	-	-	-	-	-	-	-

[1]n=36 spat of each grade; determined by direct chromosome count.
[2]Diagonal mesh size of screens used for grading.
[3]These oysters were selected for breeding experiments.
[4]This consisted of 4 diploid/triploid mosaics, 1 diploid/triploid/tetraploid mosaic and 1 aneuploid/triploid mosaic. The aneuploid had 23-27 chromosomes.
[5]This consisted of 8 diploid/triploid mosaics and 1 aneuploid/triploid mosaic. The aneuploid had 23-27 chromosomes.

Table 5

Method 2 - The fertilisation and survival of *Saccostrea commercialis* larvae[1] in January 1996.

Treatment duration	Fertilisation	Survival[2]	
(min)	(%)	Day 3	Day 6
20	96	1.1×10^6 (38)	385 000 (13)
30	94	0.8×10^6 (28)	420 000 (14)

[1]Eggs (2.9 million) stocked in a 10 000 l tank at 25°C and surviving larvae restocked in a 1 000 L tank after the first water change on day 3.
[2]Percentage survival shown in brackets.

Table 6

Method 2 - The chromosome numbers of Sydney rock oyster *Saccostrea commercialis* larvae in January 1996.

	Chromosome number (% larvae)							
	<18	18-22	23-27	28-32	33-37	38-42	43-47	48-52
20 min duration[3]								
Day 0[1]	6	41	15	27	4	4	2	0
Day 6[2]	-	13	32	33	13	4	5	-
30 min duration[3]								
Day 0[1]	0	48	23	18	2	8	0	2
Day 6[2]	-	11	34	29	11	7	8	-

[1]n=50 larvae, determined by direct chromosome count.
[2]n=2 mixed samples of larvae, determined by flow cytometry. Care should be taken in interpreting these results, ploidy levels appear to have shifted to a higher ploidy when compared to day 0 results.
[3]Exposure time to 1 mg CB/L.

Table 7

Method 2 - The total number of Sydney rock oyster *Saccostrea commercialis* spat in each grade and their chromosome number in June 1996.

Grade[2] (mm)	Number of spat	Chromosome number[1] (% spat)							
		18-22	23-27	28-32	33-37	38-42	43-47	48-52	mosaic
15.6-25.5	639	83	0	13	0	0	0	0	4[4]
10-15.6[3]	2194	75	0	13	0	0	0	0	13[5]
5.7-10[3]	1894	100	0	0	0	0	0	0	0
<5.7	2713	75	0	0	0	0	0	0	25[5]

[1]n=24 spat for the two larger grades and 12 spat for the two smaller grades; determined by direct chromosome count.
[2]Diagonal mesh size of screens used for grading.
[3] Square mesh used for grading.
[4]This was a diploid/triploid mosaic.
[5]This included 1 diploid/triploid mosaic and 2 diploid/aneuploid mosaics. The aneuploid had 23-27 chromosomes.

Table 8

Method 2 - The fertilisation and survival of *Saccostrea commercialis* larvae after tetraploidy induction in February 1997.

	Tank	
	1	2
No of females used	8	9
No of males used	4	4
No of eggs stocked	25.5×10^6	37.5×10^6
Fertilisation (%)	89	93
Survival		
Day 2 (21/2/97)	10.06×10^6	13.20×10^6
Total number of larvae put to set	0.510×10^6	0.518×10^6
Total number of spat set (18/4/97)	0.450×10^6	0.295×10^6
Total number of spat (2/6/97)		
>3.00 mm	13 000	13 000
1.25-3.00 mm	27 000	23 000

Table 9

Method 2 - The chromosome numbers of Sydney rock oyster *Saccostrea commercialis* larvae after tetraploidy induction in February 1997.

	Chromosome number (% larvae)							
	<18	18-22	23-27	28-32	33-37	38-42	43-47	48-52
Tank 1								
Day 0[1]	3	53	11	20	0	13	-	0
Day 6[2]	-	68	-	25	-	7	-	-
Tank 2								
Day 0[1]	10	55	12	13	0	10	0	0
Day 6[2]	-	80	-	15	-	5	-	-

[1]n = 60 larvae, determined by direct chromosome count.
[2]n = three samples of larvae, determined by flow cytometry. The actual tetraploid level may be 3% lower, because the background reading with sperm (haploid) samples produces ‘tetraploid’ peaks of ≥3%.

Table 10

Method 2- The chromosome number of Sydney rock oyster *Saccostrea commercialis* spat three and a half months (June 1997) after tetraploidy induction in March 1997.

	Chromosome number (% spat)[1]							
Grade	<18	18-22	23-27	28-32	33-37	38-42	43-47	48-52
Tank 1 small	-	96	-	3	-	1	-	-
Tank 1 large	-	94	-	5	-	1	-	-
Tank 2 small	-	96	-	3	-	1	-	-
Tank 2 large	-	87	-	11	-	2	-	-

[1]Determined by flow cytometry; n = 150 spat grouped for sampling per grade; Small spat are >1.25 mm and <3.00 mm and large spat are >3.00 mm. The actual tetraploid level may be 3% lower, because the background reading with sperm (haploid) samples produces ‘tetraploid’ peaks of ≥3%.

Table 11

Method 3 - Larval rearing performance and fertilisation details of tetraploid induced Sydney rock oyster *Saccostrea commercialis* larvae in February - March 1998.

	Replicate											
	1	2[1]	3	4	5	6	7[2]	8	9	10	11	12
No. of female broodstock	3	3	3	3	3	3	2	2	2	7	7	7
No. of male broodstock (pooled sperm)	11	11	11	11	11	11	6	6	6	6	6	6
pre-fertilisation[3] (%)	3	0	1	0	0	2	0	0	0	0	0	0
fertilisation (%)	99	100	100	91	97	98	100	100	98	96	98	100
no. of eggs stocked (x 10^6)	31.4	0.12[1]	21.7	32.7	17.3	12.7	4.7[2]	34.0	25.6	37.5	43.2	53[2]
Survival (x 10^6)												
Day 2	8.5	-	4.4	6.5	4.4	2.2	-	7.4	3.8	10	9.7	-
Day 4	8.5	-	3.2	5.4	2.9	2.5	-	4.8	2.8	8.8	7.1	-

[1]Tank 2 was dumped on day 1 as the number of larvae was very low.
[2]Larvae dumped on day 2 as no tetraploids were found in day 0 chromosome counts.
[3]Fertilisation before the addition of sperm, using naturally spawned females in the presence of males.

Table 12
Method 3 - The chromosome numbers of Sydney Rock oyster *Saccostrea commercialis* larvae of each treatment after tetraploidy induction in February - March 1998.

	Chromosome number (% larvae)					
Treatment	<18	18-21	22-24	25-34	35-44	45-64
Day 0[1]						
7	12	48	15	25	0[2]	0
8	15	28	17	37	3	0
9	10	45	20	20	5	0
10	17	37	10	33	3	0
11	12	35	15	35	2	2
12	18	52	7	23	0[2]	0
Day 5[3]						
1	-	13	-	82	5	-
3	-	38	-	60	3	-
4	-	33	-	64	3	-
5	-	28	-	69	3	-
6	-	52	-	44	4	-
8	-	83	-	16	1	-
9	-	51	-	47	2	-
10	-	80	-	19	2	-
11	-	33	-	65	2	-

[1]Ploidy classification from Nell *et. al.* (1996), n=90 larvae; determined by direct chromosome count at day 0. No counts were attainable for replicates 1 to 6 due to poor slide quality.
[2]Larvae from this tank were dumped on day 1 as no tetraploids were present.
[3]n = several thousand larvae used for ploidy determination by flow cytometry. A background tetraploidy level of up to 3% with flow cytometry is found in diploid controls.

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