Commercialisation of triploid Sydney rock and Pacific oysters. Part 1: Sydney rock oysters

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Part 1: Sydney rock oysters

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OBJECTIVES:

a) To produce a large number (300 000 of each type) of triploid and diploid Sydney rock oyster spat (from the same batch of eggs) for farming experiments described below.

b) To refine triploidy induction techniques for the Sydney rock oyster to enable commercial hatcheries to consistently produce a high yield of triploids. To investigate specifically caffeine as a replacement for the chemical cytochalasin B (CB) as a triploidy inducing agent. Cytochalasin B could be a potential health hazard to hatchery staff.

c) To evaluate the farming of triploid oysters against diploid oysters from the same batch of eggs by approximately 12 commercial oyster farmers (nominated by industry organisations) in major NSW oyster growing areas.

d) To make monthly comparisons of the meat condition of diploid and triploid oysters in four estuaries for 12 months.

e) To compare the susceptibility to winter mortality between stick and single seed in one estuary on the south coast of NSW.

NON TECHNICAL SUMMARY:

Diploids oysters have two sets (2 n) of chromosomes with 10 in each set, i.e. a diploid oyster has 20 chromosomes. The eggs released in spawning have four sets of chromosomes (4 n) and the sperm only one set (1 n). In the development of diploid larvae two sets of polar bodies are extruded to shed excess chromosomes after fertilisation. The first polar body contains two sets (2 n) of chromosomes and the second polar body one set (1 n), these are extruded in meiosis I (M I) and meiosis II (M II) respectively. Once both polar bodies have been extruded the one cell zygote is diploid (2 n). In this study the extrusion of the second polar body was chemically blocked (Fig. 1). Therefore the triploids used for this study were M II triploids. Experimental batches of M I triploids have been produced by other researchers, but this is more difficult than the production of M II triploids.

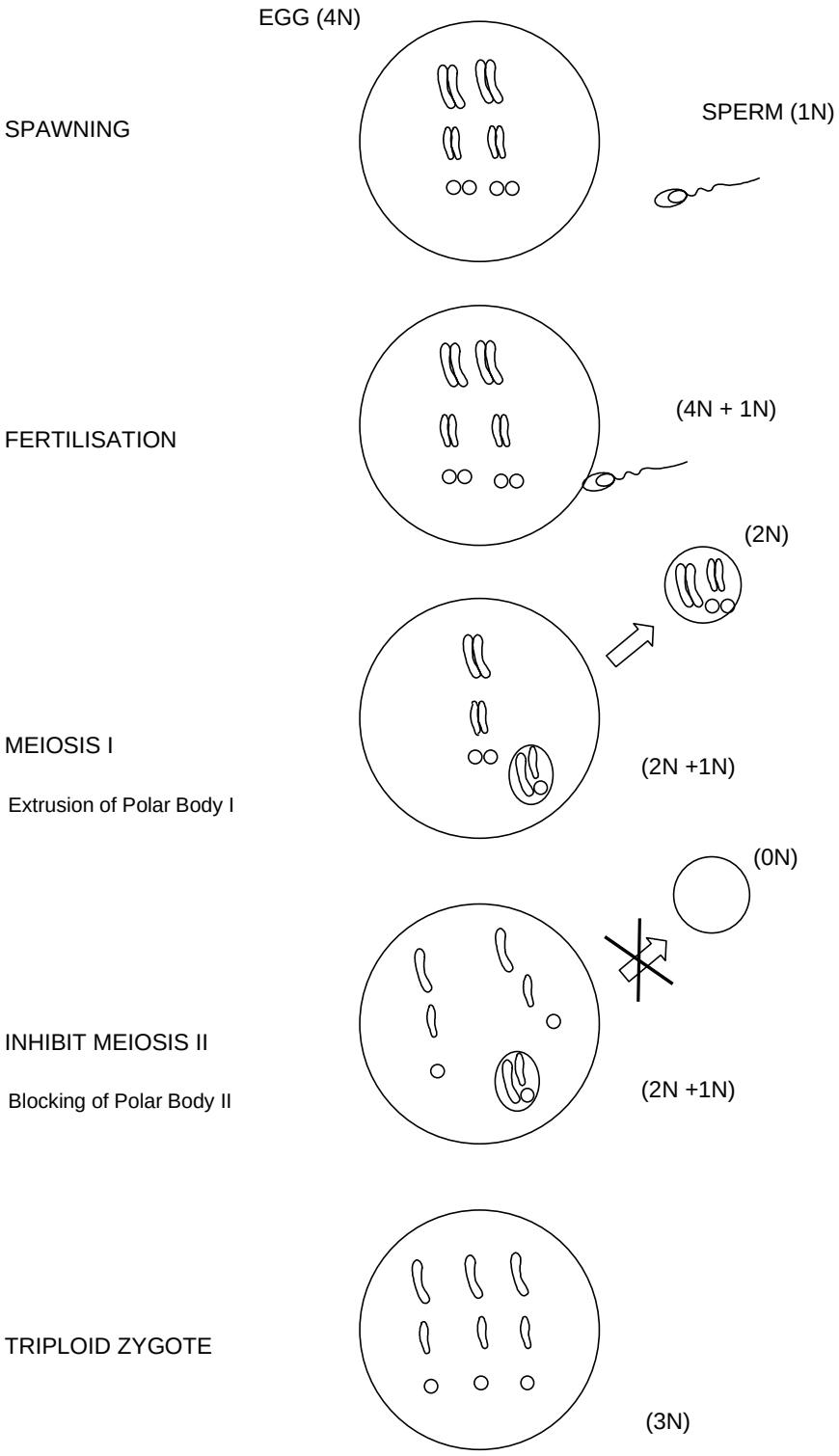

Fig. 1 Triploid induction in oysters

Major achievements

The objectives of this project were met and additional useful information was also generated.

- Eggs from naturally spawned Sydney rock oysters *Saccostrea commercialis* were used to determine the appropriate stage of development after fertilisation for inducing triploidy and to compare the effectiveness of two treatments, cytochalasin B (CB) and 6-dimethylaminopurine (6-DMAP) in dose optimisation trials. The CB treatment resulted in greater survival and triploidy percentage than 6-DMAP in Sydney rock oysters. CB has been proposed for registration for use in aquaculture in Australia.

- Techniques were developed to pre-determine the gender of live oysters to enable controlled and separate sex natural spawnings for induction trials. Eggs from natural spawnings were found to yield more viable larvae and better triploidy induction success than those from strip spawnings.

- Growth and mortality of triploid (88% triploidy) Sydney rock oysters *Saccostrea commercialis* were compared to sibling diploids at 13 oyster farms in ten estuaries in New South Wales (NSW). Although results varied between farms, after 2-2½ years on commercial oyster leases triploids were on average 30.7% heavier and 8.6% greater in shell height than sibling diploids. In general, triploids appeared to grow faster relative to diploids at higher water temperatures. Mortality of triploid oysters was either significantly lower than, or not significantly different from that of their diploid siblings. Therefore, triploid Sydney rock oysters were found to be a commercially attractive aquaculture candidate with faster growth rates and lower mortality when grown under commercial oyster farming conditions across a range of estuaries in NSW.

- A large proportion of diploid/triploid mosaicism was detected in adult oysters. This was not noted in younger triploid oysters and could be due to reversion from the triploid state. Alternatively, the inconsistency may have resulted from the different methods used for measuring chromosome numbers. For example, the analysis of groups of young oysters, as opposed to individual analyses in older animals, does not distinguish mosaic individuals.

- Diploid and triploid Sydney rock oysters *Saccostrea commercialis* were grown at seven sites in New South Wales (NSW) and exposed to the winter mortality parasite *Mikrocytos roughleyi* over two consecutive winters from July 1994 to December 1996. Triploids showed a higher survival than diploids. Over the second winter/spring, average cumulative mortality across all sites, of diploids was 35.0% (range 6.7-76.8%) compared to only 12.2% (range 4.0-18.1%) for triploids. This reduction in mortality during the second year of culture on leases could provide the Sydney rock industry in NSW with significant improvements in profitability.

- The response of triploids and diploids to winter mortality under commercial growing conditions for both tray and stick culture demonstrated clearly that tray oysters are no more susceptible to winter mortality than stick-grown oysters. Thus farmers can use tray cultivation in Merimbula River without the worry of an increased risk of oyster kill due to winter mortality. This effect may need confirmation in other estuaries affected by winter mortality before it is assumed to be general.

- The relative performance, in terms of meat condition, of triploid compared to diploid Sydney rock oysters over 13 months varied among five widely distributed sites in New South Wales. From March to December (autumn to the first month of summer), triploid condition indices were higher, or not significantly different from those of diploids at all sites. Condition indices of triploids were higher than those of diploids from May to November/December at four of the five sites. A higher condition index of triploids became apparent later in the sampling period for the Lake Pambula site (the coldest location) in comparison to the remaining four sites.

- Triploid Sydney rock oysters were susceptible to brown discolouration of the gonad surface. Discolouration occurred in localised areas of the gonad and was not correlated to condition index except for triploids at Lake Pambula. As discolouration was less noticeable during cooler months of the year, thus coinciding with the generally superior meat condition of triploids relative to diploids during winter and spring, triploids remain a viable winter crop for farmers throughout New South Wales. Triploidy extends the marketing season for Sydney rock oysters as they lose condition slower during autumn than their diploid siblings that spawn out.

- In another 2 year growout trial, triploid oysters from two initial size grades grew faster on a mean whole weight basis than sibling diploids, irrespective of initial size. That is, small grade triploids will reach market size (40 - 60 g) earlier than large grade diploids. However, initial size grade was found to have a significant effect on growth for both diploids and triploids. The demonstrated effect of initial size grade on subsequent growth of both diploid and triploid oysters is of significant commercial value to hatchery and nursery operators as well as growers of single seed oysters.

- Oysters mass selected for faster growth rate were produced (with independent funding) so that selected lines could be used for triploidy induction. The intention is to investigate the potential to combine the growth rate benefits from triploidy and mass selection.

- This project (93/151) has demonstrated the value of triploids in improving growth and survival rates of Sydney rock oysters in a wide range of waterways in NSW. Meat condition is improved in the critical cooler months without major problems with meat discolouration in those months. Improved resistance to winter mortality is another very important advantage. The technology exists to produce commercial scale batches (>75% triploidy) of triploid oysters in the hatchery which can be transferred to the private hatchery sector as it develops. The project has provided farmers in a wide range of waterways with direct experience in growing triploids and as such has greatly improved opportunities for commercialising triploids in NSW.

1. BACKGROUND

Oyster farming formed a significant component (about 13%) of the estimated total value of the Australian aquaculture industry in 1996/97 (Brown et al., 1997). The major oyster industries are in NSW ($28 million) and Tasmania ($19 million) but many farmers need to improve product quality and/or reduce production costs to stay competitive. The major species produced in NSW is the Sydney rock oyster while the Pacific oyster accounts for most of the production in Tasmania (and South Australia; $4 million in 96/97; Brown et al., 1997). Oyster farmers worldwide encounter serious problems with continuity of marketing because when oysters spawn they lose meat weight and increase in water content, i.e., they are in poor condition. It may be several months before spawned oysters recover peak meat condition. In contrast some markets suffer consumer resistance to oysters which are in an advanced stage of sexual maturation. One method of overcoming both these problems is to produce triploid oysters (3N chromosomes per cell). Triploids in contrast to normal diploid oysters (2N) do not commit as much of their energy budget to gonad maturation and are much less likely to spawn and hence lose condition (Cox et al., 1996). Triploid Sydney rock and Pacific oysters are well accepted by consumers and taste panels and grow faster than diploids (Nell et al., 1994; Maguire et al., 1998 a,b). Pacific oysters are often farmed as an exotic species; the resultant spatfall can be considered to be undesirable from both an environmental and farm management viewpoint. Being virtually sterile, triploids are much less likely to produce spatfall.

In a joint FRDC funded study between NSW Fisheries and the University of Tasmania (89/63; 1989-92), Drs Nell and Maguire showed that faster growth rates could be obtained with triploid Sydney rock oysters *Saccostrea commercialis* in NSW (about 40% faster) and triploid Pacific oysters *Crassostrea gigas* in Tasmania (>20% faster), compared with their diploid siblings. Triploidy also overcame the problem of poor post spawning meat condition in Summer - Autumn in Tasmania; triploid Pacific oysters maintained a condition index advantage over diploids for more than 200 days post spawning. Triploid Pacific oysters also exhibited better shell shape. The main objective of providing "fatter" Sydney rock oysters was partly achieved. After having reached market size the condition of triploid oysters was always equal to, but more commonly, significantly greater than their diploid counterparts. Therefore the farming of triploid Sydney rock oysters would assist the NSW oyster industry in its drive to ensure a consistently high quality product.

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2. NEED

Hatchery phase

A major impediment to commercial use of triploids is that induction methods usually produce a mixture of triploids and diploids. If harvested in the post spawning period, the diploids within a batch of "triploids" may be unmarketable and when opened by the processor would represent an economic loss as well as adversely affecting the product image. Hatcheries in Tasmania have experienced highly variable percentage triploidy results while attempting to meet the demand from farmers for several million triploid Pacific oyster spat, generated by the FRDC research program 89/63. In the above study we obtained only about 75% triploidy despite getting a world authority on triploids, Sandra Downing from University of Washington, to produce the triploid Pacific oysters in Tasmania. While a higher percentage was obtained with Sydney rock oysters (92% of spat were triploid) other batches exhibited much lower triploidy levels. Even at a research level the variability among replicate batches has been very high (Nell et al., 1996). Possible reasons include condition of broodstock, spawning method, choice of temperature, stress time and stressor concentration, type of stressor and sperm quality. The actual measurement of percentage triploidy (flow cytometry) is, however, quite reliable in New South Wales and Tasmania although it is not ideal for identifying mosaics (oysters with individual cells differing in number of chromosomes).

The recommended method for inducing triploidy in Pacific oyster embryos is to use a fungal metabolite cytochalasin B (CB), however being carcinogenic at high concentrations, this can be hazardous to hatchery staff and could harm the "clean image" of aquaculture products. High mortality of embryos using this method can also be a problem (Allen and Bushek, 1992). However, some promising initial results have been obtained by inducing triploidy in oysters with 6-dimethylaminopurine (6-DMAP; Desrosiers et al., 1993; Gérard et al., 1994). We compared the suitability of alternate methods of induction (eg. CB and 6-DMAP) after finding the optimum treatment with each method.

When oyster eggs are spawned they contain four sets of chromosomes (4N). Two reductions in chromosome number subsequently occur as two polar bodies are ejected in sequence in meiotic divisions. Each involves a halving of chromosome number. Hence, at Meiosis 1, 2N chromosomes are ejected while at Meiosis 2, 1N chromosomes are ejected leaving 1N in the egg which in combination with the 1N complement from the sperm cell produces a 2N embryo (diploid). Stress can be applied to inhibit release of either of the polar bodies. Either approach will produce triploid embryos. The standard approach for Pacific oysters has been to inhibit the release of the second polar body. However, in theory, blocking of the first polar body should lead to greater heterozygosity (genetic variation) (Beaumont and Fairbrother, 1991) and preliminary work has indicated better performance of Meiosis 1 triploids than Meiosis 2 triploids (Hawkins et al., 1994). In this project we compared the performance of Meiosis 1 and Meiosis 2 triploid Pacific oysters in the hatchery, nursery and grow-out phases.

The Sydney rock oyster study concentrated on Meiosis 2 triploidy induction. Here the primary aim was to supply Sydney rock oyster farmers with triploid spat at an early stage in the project. The large increase in growth rates (about 40%) that was obtained with M2 triploid oysters over diploid oysters in the previous study, showed that M2 triploids would be adequate. In addition, preliminary trials with the production of M1 triploid Sydney rock oysters indicated extremely high mortality of larvae.

Grow-out phase

As oysters are farmed at a range of locations and under a variety of environmental conditions in both NSW and Tasmania, there was a need to evaluate triploid oysters in a wider range of

waterways than was possible in the previous project (89/63). For example Pacific oysters needed to be evaluated in South Australia and in Sydney rock oysters in growing areas outside of the central coast of NSW.

Other aspects

Fast growing triploid oysters and the adoption of the single-seed culture technique, are both promising developments for the New South Wales oyster industry, which has declined from a peak of around 147 000 bags (1 200 oysters/bag) in the late 1970s to around 85 000 bags at present (1990- 1997). The combined use of these two new techniques can revitalise the industry, by reducing production costs and improving both meat and shell quality. Triploid oysters generally have a more consistent meat quality and the use of the single-seed culture technique produces more uniform cup-shaped oyster shells.

Some farmers on the south coast of NSW believe that single seed oysters are more susceptible to winter mortality than stick oysters. This disease is a regular and major killer of oysters in many areas of NSW. As triploids are produced in hatcheries as single seed, farmers would have to be convinced that they don't take extra risks in growing them. Thus this study included an experiment designed to compare the susceptibility of single seed oysters compared with stick oysters to winter mortality.

Commercialisation

This project provided the basis for full and immediate commercialisation of triploid Sydney rock oysters. It evaluated their performance throughout NSW and informed the NSW and southern Queensland oyster industries of the costs and results which could be expected when using triploid oysters. It also provided 12 widely distributed oyster businesses with hands-on experience in their use.

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3. OBJECTIVES

(a) To produce a large number (300 000 of each type) of triploid and diploid Sydney rock oyster spat (from the same batch of eggs) for farming experiments described below.

Triploidy Induction
A large batch of sibling diploid and meiosis II triploid oysters was produced in February 1994 in Port Stephens, NSW. Triploidy was induced by exposure of fertilised eggs to 1.25 mg/L cytochalasin B dissolved in 0.1% dimethylsulphoxide (DMSO) in filtered sea water for 15 minutes. Treatment was initiated when 50% of zygotes had extruded the first polar body. Following exposure, eggs were rinsed in filtered sea water and resuspended in 0.1% DMSO for 15 min before being stocked directly into 20 000 L larval rearing tanks.

Ploidy determination
Ploidy of triploid spat was measured prior to stocking the experiment in December 1994 by flow cytometry of three replicate groups of 70 spat for slow growers and five replicate groups of 40 spat for fast growers. In order to assess any changes in ploidy level during the course of the experiment, random samples of five oysters per triploid replicate (ie 30 oysters for each triploid grade) were sampled in December 1996. Ploidy of adult oysters was determined from direct chromosome counts of cell spreads prepared from gill.

Larvae rearing
Larvae were reared using standard hatchery techniques for Sydney rock oysters. Screening at water changes was conservative to allow for initial slow growth of triploid larvae following exposure to CB. Larvae were settled on ground scallop shell between days 18-22 and reared in the hatchery for 5 weeks before being transferred to outdoor upwelling units.

Spat production
Spat were held in outdoor upwelling units. Approximately 350 000 triploid (88% triploidy) and 350 000 diploid spat were produced for experimental purposes. An experiment to determine the effect of initial size on growth of diploid and triploid Sydney rock oysters was established in December 1994 (see Section 5.2). Spat for the commercialisation experiment (see section 5.3) were sold to participating farmers for 2.0 and 3.0 cents each for diploids and triploids respectively. Thirteen commercial oyster farmers in eleven different estuaries throughout NSW from Pambula Lake to Hastings River were each sold 25 000 diploid and 25 000 sibling triploid spat between July and November 1994.

b) To refine triploidy induction techniques for the Sydney rock oyster to enable commercial hatcheries to consistently produce a high yield of triploids. To investigate specifically caffeine as a replacement for the chemical cytochalasin B (CB) as a triploidy inducing agent. Cytochalasin B could be a potential health hazard to hatchery staff.

A wide range of chemical stressors [cytochalasin B (CB), calcium, caffeine and chemical 6-dimethylaminopurine (6-DMAP)], physical stressors (heat and hydrostatic pressure) and a combination of chemical and physical stressors have been used experimentally for triploidy induction in oysters. In this study only the chemicals (CB and 6-DMAP) were evaluated. Experimental details of triploidy induction experiments are shown in Section 5.1. In our initial attempts at triploidy induction with cytochalasin B (CB) at either 0.5 or 1.0 mg/L (1.0 or 2.1 µM) in 1990, only one out of five attempts produced larvae with ≥75% triploidy. These results were not commercially attractive, because batches of spat with <75% triploidy could not be sold as triploids.

Gamete production
For all triploidy induction experiments, Sydney rock oyster broodstock was obtained directly from oyster leases in NSW. Although 'strip' spawning is the recommended method for obtaining eggs for triploidy induction, it was found that there was much greater variability in the speed of egg development in 'stripped' eggs than those from natural spawnings. Strip spawning in Sydney rock oysters also produces fewer viable larvae than natural spawnings. To avoid uncontrolled fertilisation of naturally spawned eggs, males and females were placed on separate spawning tables. The sex of individual oysters was determined by drilling the dorsal region of the left valve and taking a biopsy of the gonad for microscopic examination of the gametes; the shell hole was then sealed using "Plasticine". Oysters were induced to spawn using standard induction techniques including the addition of stripped sperm to the spawning table. These sperm were inactivated, to avoid uncontrolled fertilisation using low level microwave irradiation until no motility was observed.

Triploidy Induction Experiments
Cytochalasin B (CB) and 6-dimethylaminopurine (6-DMAP) were compared in dose optimisation trials. Induction should commence at 50% first polar body (PB1) extrusion in eggs (approximately 17-19 min post-fertilisation at 25^{o}C). By day 5 the highest triploidy percentage and yield (number of triploid larvae/100 fertilised eggs) were achieved in the ranges of 0.75-1.5 mg CB/L (1.6-3.1 µM CB) or 200-400 µM 6-DMAP (32.6-65.3 mg 6-DMAP/L). However, CB treatment resulted in greater survival and triploidy percentage than 6-DMAP in Sydney rock oysters.

Discussion
The use of 300 and 400 µM 6-DMAP for triploidy induction achieved only 40 and 57% triploidy respectively in day 5 larvae compared to 78 and 76% with 1.0 and 1.25 mg CB/L respectively. In Sydney rock oysters, triploidy induction with CB gives higher triploidy percentages than 6-DMAP. The use of 1.0 mg CB/L at 50% first polar body formation in eggs obtained from naturally spawned females is a reliable technique for triploidy (≥75%) induction in Sydney rock oysters.

Unfortunately, physical stressors are not effective for triploidy induction in oysters. All triploidy induction chemicals are dangerous and the Material Safety Data Sheets available from chemical suppliers should be read carefully before use and all Safety Precautions Taken. Although chemicals such as caffeine, 6-DMAP and CB are toxic, their use in ploidy manipulation of bivalves involves a single, brief (10-40 min) exposure of fertilised eggs to the relevant chemical. As treatment only involves exposure of the zygote, no chemical residues need to be considered for the adult bivalves used for human consumption. Neither 6-DMAP or CB have been registered for use by the National Registration Authority for Agricultural and Veterinary Chemicals (NRA) in Australia. However commercial hatcheries can apply for a Minor Use Permit for 12 months and reapply annually. Applications should be submitted to:

National Registration Authority
PO Box E240
Kingston ACT 2604
Tel: (02) 62723797
Fax: (02) 62724753

Cost of triploid spat production
The extra cost of triploidy induction chemicals per spat sold is negligible. In 1998/99 NSW Fisheries sold both diploid and triploid Sydney rock oyster spat for 2 cents/spat. The higher mortality of larvae to Day 5 is not of great concern as there is no cost involved in stocking a tank at higher density to overcome this. However, the ripeness of the broodstock used for triploidy induction is of even greater importance than for the production of diploid spat. The criteria of ≥75% triploidy for the sale of triploid spat is a very arbitrary cut off point. It does not relate to the

economics of farming triploid oysters, it is the highest triploidy percentage that a hatchery can reliably and repeatedly achieve.

An estimate of the economic benefits of farming single-seed triploid Sydney rock oysters
Triploid oysters grow faster than traditional diploid oysters, whose three to four year production cycle involves considerable costs of establishment and production. By reducing the production time, triploid oysters offer a means to reduce costs. The reduction in costs is estimated to be about $2 per bag for each month by which production is reduced, but at least 6 months reduction would be required to offset the initial costs involved in switching from traditional natural stock to hatchery produced triploids. Additional benefits from selective breeding would include increased growth rates and disease resistance, as well as constant supply of spat throughout the year and consequent elimination of any off-season lulls in supply.

c) To evaluate the farming of triploid oysters against diploid oysters from the same batch of eggs by approximately 12 commercial oyster farmers (nominated by industry organisations) in major NSW oyster growing areas.

Growth and mortality
Growth and mortality of triploid Sydney rock oysters, *Saccostrea commercialis* were compared to those of sibling diploids at 13 oyster farms in ten estuaries in NSW (see section 5.3). Although results varied between farms, after 2-2½ years on commercial oyster leases triploids were on average 30.7% heavier and 8.6% larger in shell height than sibling diploids. Seven of the 13 farms had triploids with a mean weight of at least market size (40 g) after 2-2½ years while no farms had diploids of the same mean weight. The growth advantage of triploids began to be expressed at specific sizes rather than ages, specifically at a mean whole weight above 5-10 g or shell height of 30-40 mm. In general, triploids appeared to grow faster relative to diploids at higher water temperatures. Mortality of triploids was significantly ($p<0.01$) lower than that of diploids at six of the 13 farms and did not differ ($p>0.05$) at six of the remaining seven farms. Triploid Sydney rock oysters were found to offer significant commercial advantages over diploid oysters because of faster growth rates and lower mortality when grown under commercial oyster farming conditions across a range of estuaries in NSW.

Much of the variation in this study may be attributable to differences in water temperatures. This is most likely due to the relatively greater contribution by diploids of energy reserves to gametogenesis at higher temperatures. However, growth of both diploids and triploids at Brisbane Waters, Lake Merimbula and Hawkesbury River appears to have been influenced by an additional factor, possibly food availability. The unusually large difference (98.9%) between diploids and triploids at the Hastings River site is partly due to the poor growth of diploids. Oysters at this site were grown subtidally which may account for the faster growth of triploids at this site compared to other sites. However, growth of diploids appeared to be retarded even before they reached a size at which we would expect gametogenesis/spawning to affect their growth.

Survival through outbreaks of winter mortality
Diploid and triploid Sydney rock oysters, *Saccostrea commercialis,* were grown at seven sites in New South Wales (NSW) for 25 to 28 months and exposed to the parasite *Mikrocytos roughleyi* over two consecutive winters during the period August-November 1994 to December 1996. Triploids showed a higher survival than diploids. Over the second winter/spring average cumulative mortality of diploids across all sites, was 35.0% (range 6.7-76.8%) compared to only 12.2% (range 4.0-18.1%) for triploids. This reduction in mortality during the second year of culture on leases, combined with the growth and condition advantages that triploidy confers, could provide the Sydney rock oyster industry in NSW with significant improvements in profitability.

The high mortality of wild-caught diploids at the Brisbane Waters site during the first winter season (1995) compared with relatively low hatchery diploid and triploid mortality, may be attributed to the greater mean size of wild-caught oysters. Larger oysters are known to be more susceptible to winter mortality. The difference in susceptibility of diploids and triploids to winter mortality was most pronounced at this site, and greater in 1996 than in the 1995 winter. Despite the cumulative mortality of triploids (16.9%) being similar to that of triploids at the other sites (mean: 12.2%; range 4.0-18.1%), hatchery diploids at this site were severely affected by the parasite (76.8% cumulative mortality). Oysters at Brisbane Waters were not moved over winter to avoid infestation (in contrast to oysters at both Georges River sites and Tilligerry Creek) accounting for the higher mortality of diploids. However, this does not explain the relatively low mortality of triploids at Brisbane Waters. A similar trend of greater triploid survival relative to diploids through outbreaks of winter mortality was found for all sites: average cumulative mortality over winter/spring, 1996 of diploids across all seven sites was 35.0% (range 6.7-76.8%) compared to only 12.2% for triploids (range 4.0-18.1%) despite farmers at three of the sites using standard disease management strategies (i.e. moving stock during winter/spring). Average mortality of triploids at the eight unaffected sites in the associated farming study was 6.1% during the same winter/spring period and was 7.0% for diploids. Triploids appear to have some inherent property of that enables them to cope better than diploids with infestation by *M. roughleyi*.

d) To make monthly comparisons of the meat condition of diploid and triploid oysters in four estuaries for 12 months.

It has been evident for several years that performance of triploid shellfish in terms of their growth advantage over diploids is dependent on the environment. The advantages (or disadvantages) of triploid over diploid oysters in terms of meat condition have also been shown to vary according to growing conditions such as temperature, food availability and growing site.
The relative performance, in terms of meat condition, of triploid compared to diploid Sydney rock oysters varied among five widely distributed sites, [Hastings River, Tilligerry Creek (Port Stephens), Hunter River, Georges River and Lake Pambula] in New South Wales (see Section 5.5). From March to December (autumn to the first month of summer) triploid condition indices were higher, or not significantly different from those of diploids at all sites. Condition indices of triploids were higher than those of diploids from May to November/December at four of the five sites. A higher condition index of triploids became apparent later in the sampling period for the southern Lake Pambula site in comparison to the remaining four sites.

Triploid Sydney rock oysters were susceptible to brown discolouration of the gonad surface. Discolouration occurred in localised areas of the gonad and was not correlated to condition index except for triploids at Lake Pambula. As discolouration was less noticeable during cooler months of the year, thus coinciding with the generally superior condition of triploids relative to diploids during winter and spring, triploids remain a viable winter crop for farmers throughout New South Wales.

e) To compare the susceptibility to winter mortality between stick and single seed in one estuary on the south coast of NSW.

The response of Sydney rock oyster *Sassostrea commercialis*, triploids and diploids to winter mortality was compared under commercial growing conditions using both tray and stick culture in Lake Merimbula. The study demonstrated that tray oysters were not more susceptible to winter mortality than are stick-grown oysters. Mortality was affected significantly only by growing height. Thus farmers can use tray cultivation during winter at this location without having an increased risk of oyster kill. This result will need to be assessed in other estuaries along the central and southern NSW coast before the conclusions can be applied generally.

4. COMMERCIAL EVALUATION OF TRIPLOID SYDNEY ROCK OYSTERS

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Studies on triploid oysters in Australia: Evaluation of cytochalasin B and 6-dimethylaminopurine for triploidy induction in Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley).

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Short running title: Triploidy induction in Sydney rock oysters

Abstract. Naturally spawned Sydney rock oysters *Saccostrea commercialis* were used to determine the appropriate stage of development for inducing triploidy and to compare the effectiveness of cytochalasin B (CB) and 6-dimethylaminopurine (6-DMAP) in dose optimisation trials. Induction should commence at 50% first polar body (PB1) extrusion in eggs (approximately 17-19 min post-fertilisation at 25°C). By day 5 the highest triploidy percentage and yield (number of triploid larvae/100 fertilised eggs) were achieved in the ranges of 0.75-1.5 mg CB/l (1.6-3.1 µM CB) or 200-400 µM 6-DMAP (32.6-65.3 mg 6-DMAP/l). However, CB treatment resulted in greater survival and triploidy percentage than 6-DMAP in Sydney rock oysters.

Introduction

Induction of triploidy in Sydney rock oysters *Saccostrea commercialis* has great commercial potential, as market size triploid Sydney rock oysters hold their meat condition better throughout the year and grow approximately 40% faster than their diploid siblings (Nell, Cox, Smith & Maguire 1994). In our initial attempts at triploidy induction with cytochalasin B (CB) at either 0.5 or 1.0 mg/l (1.0 or 2.1 µM) (Downing & Allen 1987; Allen, Downing & Chew 1989) in 1990, only one out of five attempts produced larvae with ≥75% triploidy (J. A. Nell unpublished data 1990). These results were not commercially attractive, because batches of spat with <75% triploidy could not be sold as triploids.

Heat shock (Quillet & Panelay 1986; Yamamoto, Sugawara, Nomura & Oshino 1988; Yamamoto, Sugawara & Nomura 1990) and hydrostatic pressure (Chaiton & Allen 1985) will also induce triploidy in oysters but chemical stress is more effective (Downing 1987). In a direct comparison of six methods (CB, heat, calcium, caffeine, combined calcium and heat and combined caffeine and heat) CB was the most effective overall in producing viable triploids (Scarpa, Toro & Wada 1994). Desrosiers, Gérard, Peignon, Naciri, Dufresne, Morasse, Ledu, Phelipot, Guerrier & Dubé (1993) and Gérard, Naciri, Peignon, Ledu & Phelipot (1994) recommended 6-dimethylaminopurine (6-DMAP) as an alternative to CB for triploidy induction in Pacific oysters *Crassostrea gigas*, but this chemical has not been tested on Sydney rock oysters. Four replicated experiments (timing of commencement of induction, CB concentration, 6-DMAP concentration and a comparison between CB and 6-DMAP) were therefore conducted to optimise triploidy induction in Sydney rock oysters. All experiments in this study were replicated (3 replicates/treatment) whereas in many previous studies (Quillet &

Panelay 1986; Yamamoto *et al.* 1988, 1990; Desrosiers *et al.* 1993; Gérard *et al.* 1994) minimal or no replication was used.

Materials and methods

Gamete production

For all triploidy induction experiments, Sydney rock oyster broodstock was obtained directly from oyster leases in NSW (Nell 1993). Although 'strip' spawning is the recommended method for obtaining eggs for triploidy induction (Allen *et al.* 1989; Allen & Bushek 1992), it was found that there was much greater variability in the speed of egg development in 'stripped' eggs than those from natural spawnings. Strip spawning in Sydney rock oysters also produces fewer viable larvae than natural spawnings (J. A. Nell personal observation 1994). To avoid uncontrolled fertilisation of naturally spawned eggs, males and females were placed on separate spawning tables. The sex of individual oysters was determined by drilling the dorsal region of the left valve and taking a biopsy of the gonad for microscopic examination of the gametes; the shell hole was then sealed using "Plasticine". Oysters were induced to spawn using standard induction techniques (Walne 1974; Holliday 1992) including the addition of stripped sperm to the spawning table. These sperm were inactivated, to avoid uncontrolled fertilisation using low level microwave irradiation until no motility was observed.

Oysters were not conditioned as all experiments were carried out during the natural breeding season for Sydney rock oysters from January - April (Nell 1993). Only eggs and sperm from naturally spawned oysters were used, except experiment 4 where three of the four males used were strip spawned. On commencement of spawning, oysters were removed from the spawning table and placed in individual 500 ml containers of filtered seawater. Eggs were pooled and retained between 45 µm and 15 µm screens to remove extraneous material and avoid variation in development rates due to egg size. Sperm were pooled and passed through a 20 µm screen. Excess sperm was used to ensure high fertilisation rates and rapid, synchronous development.

Larval rearing

Seawater for all triploidy induction and larval rearing was filtered to 1 µm (nominal) and treated with 1 mg/l ethylenediamine tetra acetic acid (EDTA) (Utting & Helm 1985) as a precaution against metal contamination. Salinity was maintained at 35±1‰ and temperature was held at 25±1^{o}C. In order to assess any changes in triploidy percentage due to differential mortality of treated zygotes, larvae from all experiments were reared for 5 days. After treatment, each replicate was stocked at 10 embryos/ml in a 20 l aerated tank maintained at 25±0.5^{o}C. Ploidy was determined at day 0 (5 h; by chromosome counts) and at day 5 (by flow cytometry). Survival was determined at day 5. Standard feed rates (Frankish, Goard & O'Connor 1991) were used for rearing larvae, using a mixed diet of Tahitian *Isochrysis* aff. *galbana* Green, *Chaetoceros calcitrans* (Paulsen) Takano and *Pavlova lutheri* (Droop) Green. Water was totally exchanged every second day by retaining larvae on mesh screens.

Triploidy induction

Details of the four triploidy induction experiments are summarised in Table 1. CB (dissolved in 0.1% dimethylsulfoxide (DMSO)) was applied to the fertilised eggs for 15 min (Downing & Allen 1987). Controls (0 mg CB/l) were treated with 0.1% DMSO. Following exposure, eggs were rinsed in filtered seawater, resuspended in 0.1% DMSO for 15 min to remove residual CB, then resuspended in filtered seawater.

6-DMAP (stock solution dissolved in distilled water) was applied to fertilised eggs for 20 min (Desrosiers *et al.* 1993). After treatment, eggs exposed to 6-DMAP were rinsed and directly resuspended in filtered seawater.

There were three replicates (1 l seawater in a plastic beaker) per treatment in all experiments. Due to the time taken in the concentration experiments (2 and 3) for screening eggs, fertilisation of each series of replicates (10 ml sperm suspension/l) was staggered by 10 min to ensure equal exposure times of all replicates. The longest period from spawning to fertilisation (mixing sperm and eggs) was 1 h.

Experiment 1 - Effect of timing of commencement of triploid induction with CB.

The effect of timing of commencement of exposure to CB on triploid induction was tested at six different times (5, 10, 15, 20, 25 and 30 min) post-fertilisation. For each treatment (time), eggs were exposed in triplicate to 1.25 mg/l (2.6 µM) CB. At CB addition, 1 ml samples of fertilised eggs were taken and preserved in 10% formalin for later determination of the percentage of eggs which had extruded the first polar body (PB1), for measuring treatment timing. Percent polar body extrusion was chosen as the most accurate criteria for treatment initiation rather than time elapsed, because of the variable rate of development of fertilised eggs.

Experiment 2 - Effect of CB concentration on triploidy induction.

Eggs were subjected to six concentrations of CB (0.0, 0.5, 0.75, 1.0 , 1.25 and 1.5 mg/l ie 0.0, 1.0, 1.6, 2.1, 2.6 and 3.1 µM) when 50% of eggs had extruded the first polar body.

Experiment 3 - Effect of 6-DMAP concentration on triploidy induction.

Eggs were subjected to six concentrations of 6-DMAP (0, 200, 300, 400, 500 and 600 µM ie 0.0, 32.6, 49.0, 65.3, 81.6 and 97.9 mg/l) when 50% of eggs had extruded the first polar body.

Experiment 4 - Comparison of the use of CB and 6-DMAP for triploidy induction.

Triploidy induction was evaluated at the two most successful concentrations (for triploidy percentage) determined for each chemical in Experiments 2 and 3. Treatment was initiated when 50% of eggs had extruded the first polar body. CB was applied at 1 mg/l (2.1 µM) or 1.25 mg/l (2.6 µM) and 6-DMAP at 300 µM (49.0 mg/l) or 400 µM (65.3 mg/l).

Ploidy determination

Triploidy percentages in day 0 larvae were determined by chromosome counts. Direct chromosome counts on larvae can only be done readily in the trochophore stage (Gérard, Peignon & Chagot 1991; R. E. Hand, personal observation 1994); triploidy percentages in day 5 larvae were therefore determined by flow cytometry (Chaiton & Allen 1985). Techniques for chromosome preparations were adapted from Allen et al. (1989). At 4 to 6 h post-fertilisation, embryos/larvae were exposed to 0.01% colchicine for 4 h. They were then transferred to a 0.7% solution of sodium citrate for 20 min. Before fixation, Carnoy's fixative was added at a ratio of 1:10 to the sodium citrate solution to prevent cells bursting upon exposure to full-strength Carnoy's (3:1 absolute methanol: glacial acetic acid). Larvae were then fixed for 1 h in Carnoy's with the fixative being changed 3-4 times during this period.

For each replicate treatment, a suspension of the fixed larvae was dropped onto two glass slides, air-dried and stained in 10% Giemsa in phosphate buffer (pH 6.8) for 6 min. Cell counts of chromosomes of 60 larvae per replicate were counted under a microscope (400 x magnification). The following ploidy classification was applied: <18 chromosomes haploid; 18-21 diploid; 22-24 aneuploid; 25-34 triploid; 35-44 tetraploid; 45-64 pentaploid (Yamamoto *et al.* 1988). A wide range was used to account for artificial loss of chromosomes and overlapping cells to give a more realistic estimate of ploidy rather than omitting such cells from analyses (eg Guo, Hershberger, Cooper & Chew 1992a; Shen, Zhang, He & Ma 1993). Use of a narrower classification range also risks incorrect classification of polyploids as aneuploids. The percentage yield (number of live day 5 triploid larvae/100 fertilised eggs) was calculated.

Statistical analysis

Homogeneity of variance of the data was tested using the Cochran's test (Winer 1991). The data were analysed by ANOVA (Sokal & Rohlf 1981) and means were compared using the SNK procedure (Winer, Brown & Michels 1991). Data in the text and tables are expressed as means ± s.e. and those in the figures as means ± 95% confidence intervals.

Results

In all experiments fertilisation was >98% ensuring a high degree of synchrony of meiotic events. No triploids or aneuploids were detected in any control treatments. Induction of triploidy by either CB or 6-DMAP caused abnormal development in some larvae which generally resulted in greater mortality of triploids compared to diploid controls. Exposure to both chemicals slowed the development of fertilised eggs and larvae and this effect was dose-dependent. Affected trochophore and veliger larvae displayed a spiralling motion; in addition, veliger larvae from high concentration treatment groups frequently had bent hinges and/or notched valves.

Experiment 1 - Effect of timing of commencement of triploid induction with CB.

Polar body formation could be seen clearly on preserved, fertilised eggs. The average day 0 aneuploidy percentage for all treatment times ranged from 3-11%. For the treatment times tested (Table 2), highest triploidy percentages for both day 0 (80%) and day 5 (76%) larvae were achieved by the addition of CB at 20 min post-fertilisation (approximately 54% PB1 extrusion). This treatment time also produced a high yield of day 5 triploid larvae (Table 2). Day 0 triploidy percentage (80%) for the 20 min treatment was significantly higher ($P<0.05$) than all other treatments except the 30 min post-fertilisation treatment; although, at day 5 results for treatments between 15-30 min post-fertilisation were not significantly different ($P>0.05$). CB addition at meiosis 1 (5 min post-fertilisation) produced a low triploidy percentage of 63% which dropped to 41% by day 5. There was a drop in triploidy percentage from day 0 to day 5 for all treatments. Survival (Table 2) of larvae was low across all treatments with no significant effect of treatment timing ($P>0.05$). Highest survival of larvae to day 5 was 58% at 25 min post-fertilisation (71% PB1 extrusion) and lowest (25%) at 5 min (meiosis 1).

Experiment 2 - Effect of CB concentration on triploidy induction.

The average day 0 aneuploidy percentage for all CB concentrations ranged from 1-6%. The highest triploidy percentage on day 0 (Table 3) was achieved at the 1.5mg/l (75%) treatment; this was significantly higher ($P<0.05$) than at 0.5 mg/l (60%). Highest day 5 triploidy percentage (85%) and yield (56%) was at 1 mg/l CB, although there were no significant differences ($P>0.05$) for either of these criteria between groups exposed to CB. There was an increase in triploidy percentage from day 0 to day 5 for all CB concentrations. Survival (Table 3; Fig.1) to day 5 was significantly affected by CB exposure ($P=0.003$) with greater survival (89%) of controls compared with all CB treated groups. Survival (46%) at the highest dose of 1.5 mg CB/l was lower than at other CB concentrations with the difference being significant ($P<0.05$) for 0.5 and 1.0 mg/l.

Experiment 3 - Effect of 6-DMAP concentration on triploidy induction.

Day 0 triploidy percentages (Table 4) at 6-DMAP concentrations from 300-600 µM were significantly higher than those at 200 µM, however, by day 5 these differences (Table 4; Fig. 2) were no longer significant ($P>0.05$). The average day 0 aneuploidy percentage for all 6-DMAP concentrations ranged from 2-5%. 6-DMAP was toxic at high concentrations with survival to day 5 (Table 4; Fig. 2) as low as 10% at 600 µM compared with 65 and 86% for larvae at 200 µM and control larvae respectively. There was a decline in triploidy percentage between days 0 and 5 at all concentrations (Table 4). Highest triploidy percentage at day 5 was produced at 400 µM 6-DMAP. Day 5 triploidy yields at 200 and 300 µM 6-DMAP were significantly higher ($P<0.05$) than those at any of the other concentrations (Table 4).

Experiment 4 - Comparison of the use of CB and 6-DMAP for triploidy induction.

Overall, triploidy percentage was lower than in previous experiments particularly for 6-DMAP treatments. The average day 0 aneuploidy percentage for all treatments ranged from 2-6%. Comparison of CB and 6-DMAP revealed no significant difference ($P>0.05$) between the two chemicals at the concentrations tested for day 0 triploidy percentages (Table 5). However, lower survival of 6-DMAP treated larvae appeared to have caused a greater reduction in triploidy percentages between days 0 and 5 than was the case for CB treated larvae (Table 5). For example, exposure to 300 µM 6-DMAP (49.0 mg/l) produced a significantly ($P<0.05$) lower triploidy percentage in larvae (40%) at day 5 than either 1 or 1.25 mg/l CB (78 and 76% respectively), although there were no significant differences ($P>0.05$) between these treatments on day 0. Survival results of 300 and 400 µM 6-DMAP treatments to day 5 were 44 and 35% respectively compared with 59% for both CB treatments. The highest day 5 yields, triploidy percentages and survivals were achieved with 1 and 1.25 mg CB/l.

Discussion

Timing of extrusion of the first polar body in *S. commercialis* was similar to that reported for *C. gigas*; for all experiments 50% of eggs had extruded the first polar body between 17 and 19 min post-fertilisation at 25°C compared to between 15 and 20 min at the same temperature for *C. gigas* (Guo *et al.* 1992a,b; Desrosiers *et al.* 1993; Longo, Matthews & Hedgecock 1993). As for *C. gigas*, maximum triploidy induction corresponded to exposure to CB when approximately 50% of eggs had extruded the first polar body (Allen *et al.* 1989). Results from these experiments illustrate the variation in timing of meiotic events between different batches of eggs thus emphasising the importance of using a development criterion (*eg*. percent polar body extrusion) rather than elapsed time to measure treatment initiation.

The average day 0 aneuploidy percentage for all treatments across all experiments ranged from 1-11%. The high day 0 aneuploidy percentage, may have partly been caused by the rupturing and overlapping of cells in the chromosome staining technique, although it is a rather common phenomenon to find aneuploids associated with triploids (Yamamoto *et al.* 1988; Guo *et al.* 1992a). Unfortunately day 5 aneuploidy percentages were not determined because direct chromosome counting is difficult on shelled larvae (Gérard *et al.* 1991; R. E. Hand, personal observation 1994). Triploidy percentages dropped between day 0 and day 5 in CB Experiment 1, 6-DMAP Experiment 3 and the 6-DMAP treatments in Experiment 4. This may have been caused by the higher mortality in triploid larvae (Beaumont & Fairbrother 1991) or the use of different triploidy determination techniques used for day 0 (direct chromosome count) and day 5 (flow cytometry) in this study.

Day 5 triploidy percentages were not very sensitive to timing of commencement of induction (15-30 min; Table 2) or CB (0.5-1.5 mg/l; Table 3) or 6-DMAP (200-600 µM; Table 4) concentrations, but survival to day 5 was relatively sensitive to high CB (Table 3) and high 6-DMAP (Table 4) concentrations.

The optimum (Table 3) CB concentration range (0.75-1.5 mg CB/l) for triploidy induction in Sydney rock oysters is on the high side of the range (0.5-1.0 mg CB/l) recommended for Pacific oysters (Yamamoto *et al.* 1988; Allen *et al.* 1989) and much higher than that (0.25 CB mg/l) recommended by Barber, Mann & Allen (1992) for American oysters *C. virginica*. However, the optimum 6-DMAP concentration range (200-400 µM) for triploidy induction in Sydney rock oysters (Tables 4 and 5) was similar to that found by Desrosiers *et al.* (1993) and Gérard *et al.* (1994), who recommended a concentration of 300 and 450 µM respectively for Pacific oysters.

Within the optimum concentration ranges for induction, 1.0 mg CB/l or 300 µM 6-DMAP, treating eggs in 1 l suspension would cost (Sigma-Aldrich, Castle Hill, NSW, Australia) AUD$16.72 and AUD$5.53 for CB and 6-DMAP in 1994 respectively.

The use of 300 and 400 µM 6-DMAP for triploidy induction achieved only 40 and 57% triploidy respectively in day 5 larvae compared to 78 and 76% with 1.0 and 1.25 mg CB/l respectively in Experiment 4 (Table 5). In Sydney rock oysters, triploidy induction with CB gives higher triploidy percentages than 6-DMAP. The results agree with those of Gérard *et al.* (1994) in a Pacific oyster triploidy induction study. The use of 1.0 mg CB/l at 50% first polar body formation in eggs obtained from naturally spawned females should greatly improve the reliability of triploidy (≥75%) induction in Sydney rock oysters.

Although this study used more replication than other studies (Quillet & Panelay 1986; Yamamoto *et al.* 1988, 1990; Desrosiers *et al.* 1993; Gérard *et al.* 1994), it still lacked statistical power. Typical minimum significant difference (P<0.05) values ranged from 10-30%. Variation among replicates was also a major problem for Downing & Allen (1987). More replication may be needed if better resolution of optimum concentration is to be achieved. However, there were large differences in results between experiments eg. differences in survival rate to day 5 for 300 and 400 µM 6-DMAP for Experiments 3 and 4, and it is likely that differences between groups of broodstock have an important effect on triploidy induction (Allan & Bushek 1992).

Although chemicals such as caffeine, 6-DMAP and CB are toxic, their use in ploidy manipulation of bivalves involves a single, brief (10-40 minute) exposure of fertilised eggs to the relevant chemical. As treatment only involves exposure of the zygote, no chemical residues need to be considered for the adult bivalves used for human consumption. Recently, five chemicals used in ploidy manipulation (including CB and 6-DMAP) were submitted to the National Registration Authority (NRA) in Australia to be registered for use in the Australian aquaculture industry. We are also attempting to produce tetraploid (Guo & Allen 1994) Sydney rock oysters by inhibiting polar body 1 in eggs from triploids. This technique requires the use of chemicals in tetraploidy induction but should eliminate the use of chemicals in the production of triploids by crossing tetraploids with diploids.

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Table 1 Triploidy induction experiments[1] in Sydney rock oysters *Saccostrea commercialis*

Exp	Egg Density ($x\ 10^6$/l)	Chemical	Concentration		Time[3] (min)	Treatment duration (min)	No of broodstock used[2]	
			(μM)	(mg/l)			Males	females
1	5.0	CB	2.6	1.25	18-19	15	5	28
2	5.0	CB	1.0-3.1	0.5-1.5	17-18	15	4	23
3	3.8	6-DMAP	200-600	32.6-97.9	18-19	20	6	19
4	3.8	CB	2.1 & 2.6	1.0 & 1.25	17-18	15	4	50
		6-DMAP	300 & 400	49.0 & 65.3	17-18	20		

[1]All experiments were carried out at 25±1^{o}C.
[2]The ratio of female to male oysters in natural mixed sex spawnings during the breeding season is approximately 3 : 1 (n=1 621) (J. A. Nell, personal observation, 1994).
[3]Time when 50% of eggs had extruded the first polar body

Table 2 Effect of timing of commencement of triploid induction with CB in Sydney rock oysters *Saccostrea commercialis* (Experiment 1)[1]

Start of induction after fertilisation (min)	Day 0 Triploidy (min)	Day 5 triploidy[2] (%)	First polar body formation (%)	Survival[3] to day 5 (%)	Day 5 triploid yield[3,4] (%)
5	63 ± 1.7^{a}	41 ± 6.0^{a}	0 ± 0.0^{a}	25 ± 4.0	10 ± 2.0
10	56 ± 3.6^{a}	49 ± 3.2^{ab}	7 ± 1.3^{b}	45 ± 9.8	22 ± 3.6
15	65 ± 4.3^{a}	59 ± 2.9^{abc}	35 ± 1.3^{c}	39 ± 2.9	23 ± 1.7
20	80 ± 2.9^{b}	76 ± 1.9^{c}	54 ± 2.0^{d}	43 ± 4.7	32 ± 3.7
25	67 ± 1.9^{a}	59 ± 11.7^{abc}	71 ± 1.5^{e}	58 ± 9.3	36 ± 12.4
30	78 ± 1.7^{b}	73 ± 2.6^{bc}	72 ± 2.1^{e}	39± 4.0	28 ± 1.9

[1] Values are means ± SE (n=3). Within columns, means with different letters differ significantly (P<0.05).
[2] Care should be taken in interpreting these data as variances were not homogeneous (P=0.02; Cochran's test).
[3] No significant differences (P>0.05).
[4] Number of live day 5 triploid larvae/100 fertilised eggs.

Table 3 Effect of CB concentration on triploidy induction in Sydney rock oysters *Saccostrea commercialis* (Experiment 2)[1]

Cytochalasin B Concentration (mg/l)	Day 0 Triploidy (%)	Day 5 Triploidy (%)	Survival to day 5 (%)	Day 5 triploid yield[2] (%)
0	0 ± 0.0^{a}	0 ± 0.0^{a}	89 ± 7.2^{a}	0 ± 0.0^{a}
0.5	60 ± 3.9^{b}	72 ± 4.5^{b}	73 ± 4.3^{bc}	53 ± 5.4^{b}
0.75	73 ± 2.2^{bc}	75 ± 1.9^{b}	55 ± 0.9^{b}	42 ± 0.8^{b}
1.0	68 ± 1.0^{bc}	85 ± 0.4^{b}	66 ± 2.0^{bc}	56 ± 1.9^{b}
1.25	73 ± 4.9^{c}	84 ± 5.0^{b}	57 ± 5.8^{c}	49 ± 7.4^{b}
1.5	75 ± 3.5^{c}	84 ± 5.0^{b}	46 ± 3.9^{c}	38 ± 5.5^{b}

[1]Values are means ± SE (n=3). Within columns, means with different letters differ significantly (P<0.05).
Number of live day 5 triploid larvae/100 fertilised eggs.

Table 4 Effect of 6-DMAP concentration on triploidy induction in Sydney rock oysters *Saccostrea commercialis* (Experiment 3)[1]

6-DMAP concentration (µM)	Day 0 Triploidy (%)	Day 5 Triploidy (%)	Survival to day 5 (%)	Day 5 triploid yield[2] (%)
0	0 ± 0.0^{a}	0 ± 0.0^{a}	86 ± 0.9^{a}	0 ± 0.0^{a}
200	53 ± 4.2^{b}	49 ± 4.1^{b}	65 ± 3.0^{b}	32 ± 4.1^{c}
300	72 ± 3.8^{c}	65 ± 7.5^{b}	57 ± 2.3^{c}	37 ± 4.4^{c}
400	75 ± 4.4^{c}	73 ± 8.1^{b}	23 ± 2.8^{d}	17 ± 3.9^{b}
500	76 ± 2.0^{c}	64 ± 5.2^{b}	19 ± 1.1^{d}	12 ± 0.4^{b}
600	76 ± 5.5^{c}	68 ± 3.2^{b}	10 ± 0.9^{e}	7 ± 0.5^{ab}

[1]Values are means ± SE (n=3). Within columns, means with different letters differ significantly (P<0.05).
[2]Number of live day 5 triploid larvae/100 fertilised eggs.

Table 5 Comparison of the use of CB and 6-DMAP for triploidy induction in Sydney rock oysters *Saccostrea commercialis* (Experiment 4)[1]

Treatment	Day 0 triploidy[2] (%)	Day 5 Triploidy (%)	Survival to day 5 (%)	Day 5 triploid yield[3] (%)
6-DMAP 300 µM	67±1.5	40±3.7[a]	44±4.2[ab]	18±2.2[a]
6-DMAP 400 µM	81±3.9	57±9.8[ab]	35±3.3[a]	21±5.1[a]
Cytochalasin B 1 mg/l	75±3.3	78±7.4[b]	59±3.7[b]	46±4.2[b]
Cytochalasin B 1.25 mg/l	77±3.3	76±2.3[b]	59±5.4[b]	45±4.2[b]

[1]Values are means ± SE (n=3). Within columns, means with different letters differ significantly ($P<0.05$).

[2]No significant differences ($P>0.05$).

[3]Number of live day 5 triploid larvae/100 fertilised eggs

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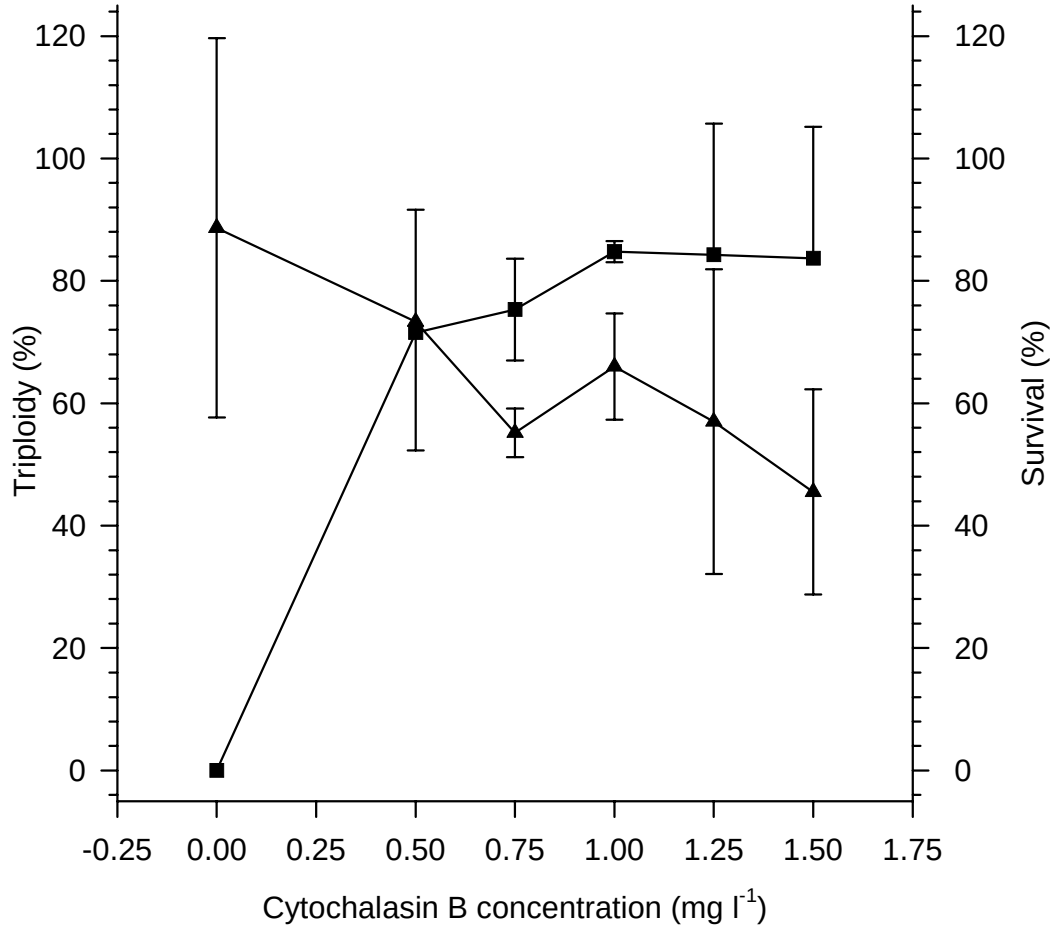

Figure 1 Effect of cytochalasin B concentration on per cent triploidy (■) and survival (▲) in 5-day-old Sydney rock oyster larvae, *Saccostrea commercialis*. Means ± 95% confidence intervals (n=3).

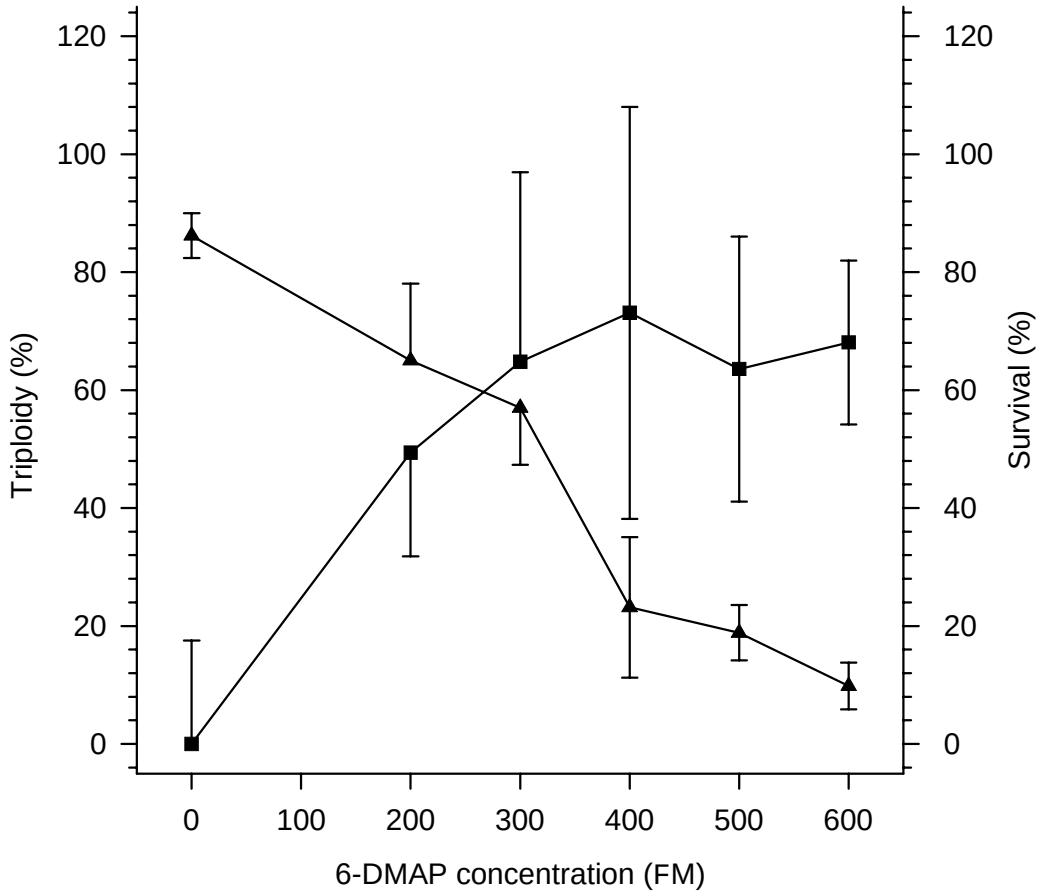

Figure 2 Effect of 6-DMAP concentration on per cent triploidy (■) and survival (▲) in 5-day-old Sydney rock oyster larvae, *Saccostrea commercialis*. Means ± 95 % confidence intervals (n=3)

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Studies on triploid oysters in Australia: effect of initial size on growth of diploid and triploid Sydney rock oysters *Saccostrea commercialis* (Iredale & Roughley)

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Abstract

In a 2 year growout trial, triploid oysters from two initial size grades grew faster (in terms of both mean whole weight and shell height) than equivalent initial size grades of sibling diploids ($P < 0.05$). Small size grade triploids caught up with and had significantly heavier ($P < 0.05$) final whole weights than large size grade diploids after 2 years' growout. Initial size grade had a significant effect on final mean whole weight and shell height for both ploidy types. After 2 years' growout, final mean whole weights (but not shell heights) of small and large diploids (35.8±0.6 g and 39.4±0.5 g respectively) were significantly different ($P < 0.05$). Small and large triploids grew at a similar rate for the first 18 months despite a significantly ($P < 0.05$) heavier final mean weight of large grade triploids (48.4±0.8 g and 61.2±0.7 g, respectively). The demonstrated effect of initial size grade on subsequent growth of both diploid and triploid oysters is of significant commercial value to hatchery and nursery operators as well as growers of single seed oysters. In addition, small grade triploids appeared to be more valuable in terms of potential growth rate than all diploid grades. There was no significant difference in the final percentage triploidy between small and large grade triploids. A large proportion of diploid/triploid mosaicism was detected in adult oysters.

Introduction

Production of triploid Sydney rock oysters, *Saccostrea commercialis* (Iredale and Roughley) is likely to be commercialised in New South Wales (NSW) following research into their production and growout trials throughout the state. Increases of up to 40% in whole weight of triploid Sydney rock oysters compared to sibling diploids were recorded. In addition, market-size triploid Sydney rock oysters in NSW maintained higher meat condition indices than diploids, particularly during winter and spring when diploids are frequently unmarketable (Nell, Cox, Smith & Maguire 1994).

Triploidy is usually induced in bivalves by inhibiting extrusion of either polar body 1 or 2 immediately after fertilisation of the egg (Beaumont & Fairbrother 1991). More recently, triploid Pacific oysters have been produced from tetraploid/diploid crosses (Guo, DeBrosse & Allen 1996). Irrespective of the method used for production of triploids, careful control of zygote development and/or fertilisation is required. This necessitates use of bivalve hatchery technology.

Hatchery production of bivalve spat results in a range of size grades from each batch. Commercial hatcheries regularly grade spat and sell them at a rate according to size grade. These values take into account the time and cost of growing a smaller grade to the same size as a larger grade as well as the concept that smaller grades may be intrinsically slower growers. The question arises as to whether small grade oysters will grow at the same rate as large grade oysters if they catch up in size. As triploids can only be produced under hatchery conditions, the question of whether different grades are of equal value in terms of growth potential is important to their commercialisation. In addition, a significant factor for commercial hatcheries to consider in

triploid production is the relative growth potential of small grade triploids compared to all diploid grades. That is, it is possible that the growth advantages of small grade triploids (due to limited gonadogenesis) may result in faster growth rates than all diploid grades. Growth potential of different grades according to ploidy level has not previously been examined in bivalves.

Considerable literature exists on the relative growth rates of different size grades of oysters and the reasons (genetic or environmental) for differences in size (Newkirk & Haley 1982, 1983; Foltz & Chatry 1986; Mason, Reid & Nell 1998). Oysters from different size grades grew at similar relative growth rates when cultured under the same conditions despite different absolute growth rates (Mason *et al.* 1998). That is, they suggest that smaller oysters were simply at a different position on the same exponential growth curve. Oysters grown under poor growing conditions (eg limited food availability, low temperatures) were temporarily stunted during the period of exposure but recovered to achieve relative growth rates comparable to oysters from a "good" growth environment when placed under the same favourable growing conditions. Similarly, Newkirk (1981) and Newkirk & Haley (1982) found that there was little relationship between the size of juvenile European oysters, *Ostrea edulis* Linnaeus, and subsequent growth rates to market size. In contrast, Losee (1979) found that *Crassostrea virginica* Gmelin larvae which reached metamorphosis earlier (ie faster growing larvae) developed into faster growing spat.

In this paper, two initial size grades of diploid and triploid Sydney rock oysters derived from the same spawning are evaluated in terms of potential growth and the ensuing management implications for commercial hatchery operators are discussed.

Materials and methods

Production of spat

A large batch of sibling diploid and meiosis II triploid oysters was produced in February 1994 in Port Stephens, NSW. Triploidy was induced by exposure of fertilized eggs to 1.25 mg/L cytochalasin B dissolved in 0.1% dimethylsulphoxide (DMSO) in filtered sea water for 15 minutes. Treatment was initiated when 50% of zygotes had extruded the first polar body. Following exposure, eggs were rinsed in filtered sea water and resuspended in 0.1% DMSO for 15 minutes before being stocked directly into 20 000 L larval rearing tanks (Nell, Hand, Goard & McAdam 1996). Larval rearing techniques are described in Hand, Nell & Maguire (1998). Spat were held in outdoor upwelling units under identical conditions until the experiment was established in December 1994. Oysters were graded in November 1994 to separate sufficient numbers for the experiment of the smallest (retained on a 3 mm mesh screen) and largest (retained on a 7 mm mesh screen) oysters of both diploids and triploids.

Ploidy determination

Ploidy of triploid spat was measured prior to stocking the experiment in December 1994 by flow cytometry (Chaiton & Allen 1985) of three replicate groups of 70 spat for slow growers and five replicate groups of 40 spat for fast growers. In order to assess any changes in ploidy level during the course of the experiment, random samples of five oysters per triploid replicate (ie 30 oysters for each triploid grade) were sampled in December 1996. Ploidy of adult oysters was determined from direct chromosome counts of cell spreads prepared from gill tissue (eg Thiriot-Quiévreux & Ayraud 1982; Allen, Downing & Chew 1989).

Experimental design and oyster management

The experiment consisted of six replicate trays of 500 oysters for small and large oysters of each ploidy level, i.e. 24 trays in total. Oyster spat were placed on commercial tarred timber and polyethylene mesh trays (0.9 m x 1.8 m) divided into 18 sections with an identical tray secured on top as a lid. Small spat were placed on 3 mm mesh until they reached a size at which they could be transferred to 6 mm mesh trays. The large size grade spat were placed directly onto 6 mm mesh trays as this would be the mesh size used for the small size grade when they reached the same size. Both grades were stocked at 50% tray coverage throughout the experiment (Holliday, Maguire & Nell 1991).

Spat were placed on a commercial, intertidal oyster lease at North Arm Cove, Port Stephens, NSW (Holliday, Maguire & Nell 1991) in December 1994. Trays were placed on the lease in a randomised block design (Sokal & Rohlf 1995) comprising two blocks (sets of lease rails) with three replicates for each treatment randomly allocated to each block. Trays were removed from the lease every 3 months and the oysters washed and dried for measurement of growth (shell height and whole weight) and mortality as well as to remove fouling organisms (including overcatch of oyster spat). Dead oysters were counted and removed from the experiment and the oyster stocking density adjusted to 50% tray surface coverage before placing trays back on the lease. During periods of heavy spat settlement in Port Stephens, trays and oysters were dried indoors for a week to kill overcatch. At each sampling period and at the commencement of the experiment a random sample of 50 oysters was measured for shell height and whole oyster weight. Growth analyses were carried out using tray means as the experimental unit, to avoid the problem of pseudoreplication (Habicht, Seeb, Gates, Brock & Olito 1994).

Due to the large size of triploid oysters in September 1996 densities could not be maintained at 50% tray coverage without removing some of the oysters. To maintain the standard stocking density, the total number of oysters was counted for each tray and reduced randomly by 50% in all treatments.

Statistical analyses

Data for shell height and whole oyster weight are displayed in Figs. 1 and 2 as means ± 95% confidence intervals (n=6). The SAS Institute Inc. (1989) GLM procedure was used to obtain estimates for the analysis of variance (ANOVA) and the STATISTICA package (Statsoft Inc. 1995) was used for the other tests. Data were analysed (with trays as the experimental unit) by a three factor (block, ploidy and size category) nested ANOVA (Table 2). Homogeneity of variance of the data was checked with the Brown-Forsythe modification of Levene's test (Brown & Forsythe 1974) which is recommended by Conover, Johnson and Johnson (1981) for its robustness and relative power compared to other tests for homogeneity of variance. A logarithmic transformation stabilised the variance. Mortality data at the completion of the experiment were compared by a 3 factor (block, ploidy, size) ANOVA after homogeneity of variances was confirmed using the Brown-Forsythe modification of Levene's test. An arcsin $x^{0.5}$ transformation was used to stabilise the variance. Ploidy data are presented as mean ± standard error and were compared between small and large triploid grades by ANOVA. An arcsin $x^{0.5}$ transformation was used to satisfy the homogeneity of variance test.

Results

Ploidy

The triploidy levels, as measured by flow cytometry, of spat at the beginning of the experiment were significantly different ($P = 0.01$) with 73.0±8.4% (n=5) and 93.3±7.6% (n=3) triploids in the large and small size grades respectively. At the end of the experiment chromosome counts of gill tissue gave slightly higher triploidy levels of 83.3±9.5% and 96.7±3.3% triploids in the large and small size grades respectively, but these initial versus final ploidy differences were not significant ($P > 0.05$). Direct chromosome counts include diploid/triploid mosaics in the percentage triploid. Similarly, flow cytometry results include mosaics in the determined ploidy level. Direct chromosome counts revealed a high percentage of diploid/triploid mosaics of 46.7±9.9% and 33.3±11.5% in the large and small size grades respectively at the end of the experiment, but these differences were not significant ($P = 0.4$).

Growth

Initial and final sizes of spat are shown in Table 1. Ploidy did not have a significant effect on initial shell height ($P = 0.95$) or weight ($P = 0.16$) but was significant for both final shell height ($P = 0.02$) and final weight ($P = 0.02$). As was expected, size grade was found to have a significant effect on initial ($P < 0.01$) and final ($P < 0.05$) mean shell height as well as initial ($P = 0.01$) and final ($P < 0.05$) weight.

Figs. 1 and 2 clearly illustrate the differences in growth over 24 months of the four types of oyster. After 10 to 11 months' growout (October/November, 1995) the small triploid grade had equalled the shell height of the large diploid grade and thereafter continued to grow at a slightly faster rate. Final shell heights of both small and large triploids (final mean heights 72.0±0.6 mm and 74.9±0.6 mm, respectively) were significantly larger than those for small and large diploids (final mean heights 66.2±0.6 mm and 67.0±0.5 mm, respectively). Post hoc comparisons showed significant differences between size categories of triploids ($P < 0.05$) in terms of mean shell weight, but not between the two size grades in diploid oysters.

The differences in growth are more pronounced when whole weights are compared (Fig. 2). Within each size grade (small or large) there appeared to be little difference between growth of diploids and triploids until September 1995 when faster growth of the triploids became apparent. After 12 to 13 months' growout (December to January 1996), the small triploids had overtaken the large diploids in whole weight. After 2 years' growout, the mean weight of oysters was found to be significantly affected by both ploidy ($P < 0.05$) and initial size ($P < 0.05$). The growth advantage of triploids over diploids was 35.6% for small and 56.6% for large grade oysters (table 1). Mean final whole weights were significantly different ($P < 0.05$) for all four treatment categories.

Mortality

Cumulative mortality of both grades of diploids and triploids throughout the experiment is shown in Fig. 3. After 2 years on leases there was no significant difference in the cumulative mortality of oysters between treatments ($P > 0.05$). High temperatures whilst stock was being dried during the summer of 1996 caused heat kill of oysters. This is reflected in the greater increase in cumulative mortality for all treatments between January 1995 and March 1996 when compared to other months.

Discussion

Although ploidy analysis at the end of the experiment showed no significant difference in percentage triploidy between the small and large grade ($P > 0.05$), initial readings revealed a significantly ($P = 0.01$) higher triploidy level in the small size grade (93% c/f 73%). In addition, a high level of diploid/triploid mosaicism was shown through chromosome counts at the end of the experiment. Early differential growth of diploid spat within the triploid batch may account for the differences in initial ploidy levels (Thompson, Wattendorf, Hestand & Underwood 1987) but does not explain the increase in ploidy during growout. Likewise, differential mortality of diploids within the triploid grades seems unlikely considering the high mortality rate of diploids that this would imply (64% for the large size grade). In fact, no significant difference was found between the cumulative mortality of diploid and triploid groups in this experiment. Flow cytometry (the method used to determine initial ploidy levels) is a less precise technique than chromosome counting, varying by 5% or more (Cozens personal communication 1994) from the actual ploidy level of a batch of oysters. It also includes diploid/triploid mosaics in the determination of ploidy when animals are analysed in groups (larvae and small spat analyses). Direct chromosome counts at the end of the experiment may therefore represent a more accurate estimate of the ploidy of both grades throughout the experiment. The percentage mosaic was unexpected and not obvious in earlier analysis by flow cytometry. Allen, Guo, Burreson & Mann (1996) report a reversion of 15 to 20% of identified triploid oysters (*Crassostrea gigas*) to a heteroploid mosaic state after a season in the field. However, as spat in the present experiment were analysed in groups by flow cytometry it is unknown whether this level of mosaic oysters was present when the experiment was established. As there was no significant difference in final ploidy between the two triploid grades, small grade triploids are of at least equal value to large grades to hatchery producers and oyster farmers in terms of percent triploid.

Weight (rather than shell height) is the major factor in determining oyster marketability and it is in weight that the advantages of triploids over diploids are most obvious (Jiang, Gang, Xu, Lin & Qing 1993; Nell *et al.* 1994). Likewise, the most marked differences in the present study were found in growth of diploids and triploids when final weights rather than shell heights were compared. Several methods for comparing growth are discussed by Francis (1996). For our purposes, ie to determine

whether both small and large grade triploid oysters will reach a larger weight than equivalent initial gradings of diploids during growout, we have used Francis' Method 1 (comparison of size at the same age). In the present study, triploid oysters grew faster than diploid oysters irrespective of the initial size grade. The effect of ploidy on growth was significant for whole oyster weight ($P = 0.02$): small grade triploids grew faster than equivalent grade diploids (triploids were 35% heavier after 2 years) and large grade triploids grew faster than equivalent grade diploids (55% heavier after 2 years). Results were similar for both grades to those reported previously for triploid Sydney rock oysters (Nell *et al.* 1994) which ranged from 31 to 41% heavier than diploids after 24 to 29 months' growth. The differences in growth of diploids compared to triploids are hardly surprising. Of greater interest is the fact that, despite being initially smaller, the small grade triploids caught up with the large grade diploids and were significantly heavier by the end of the experiment ($P < 0.05$).

To the present authors knowledge, this is the first reported comparison of growth of different size grades of diploid and triploid oysters. However, variable growth rates have previously been related to heterozygosity in bivalves (Fujio 1982; Foltz & Chatry 1986; Gentili & Beaumont 1988). More specifically, superior growth rates of triploids have been at least partially attributed to higher heterozygosity (Hawkins, Day, Gérard, Naciri, Ledu, Bayne & Héral 1994). These authors were referring to meiosis I triploids which are generally more heterozygous than both diploids and meiosis II triploids (produced in this case). If differences in relative heterozygosities exist between grades they may not be expressed in growth of oysters unless the shellfish are grown under stressful conditions (eg high density, Gentili & Beaumont 1988).

Initial size grade was found to have a significant effect on subsequent growth (mean whole weight ($P < 0.05$) and shell height ($P < 0.05$)) of diploid and triploid oysters. After two years' growout, significant differences ($P < 0.05$) were shown between mean whole weights of small and large grade oysters within both ploidy types but not between mean shell heights of diploids ($P > 0.05$). These results support the general assumption of commercial hatchery operators that the final grading of a hatchery batch of diploid spat is slower growing and therefore of lower value than the top gradings. Similarly, Losee (1979) found that hatchery reared *C. virginica* that reach metamorphosis earlier than sibling larvae achieved greater subsequent growth rates. In general, Sydney rock oysters take around 3 years to reach market size (Nell 1993). Although initial size affected triploid growth during the experiment, both small and large grade triploids reached market size (40 g) after 2 years' growout whilst both diploid grades were still slightly below marketable weight.

In conclusion, under standard farming conditions small grade diploids and triploids were slower in growth (by weight) when compared to the large grade oysters of the equivalent ploidy type. These results indicate that a lower value should be placed on hatchery produced "tail-ender" diploids. However, the small triploid oysters still had a larger mean size than both diploid grades after two years' growout. That is, small through to large grade triploid spat are of greater value to hatchery operators in terms of growth potential than all diploid grades. Small and large grade triploids were equivalent in terms of percentage triploid. These findings are of significant commercial value to hatchery/nursery operators and growers of single seed oysters.

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Table 1 Initial and final sizes[1] (shell height and whole oyster weight) of two size grades of diploid and triploid *Saccostrea commercialis* grown for 24 months at North Arm Cove, Port Stephens.

	Initial		Final[2]		Triploid growth advantage	
Oyster type	**height (mm)**	**weight (g)**	**height (mm)**	**weight (g)**	**height (%)[3]**	**weight**
Small diploid	15.9 ± 0.11	0.4 ± 0.01	66.2 ± 0.55[a]	35.8 ± 0.62[a]		
Small triploid	15.7 ± 0.14	0.4 ± 0.01	72.0 ± 0.56[b]	48.4 ± 0.80[b]	11.9	35.6
Large diploid	26.3 ± 0.48	1.6 ± 0.08	67.0 ± 0.46[a]	39.4 ± 0.45[c]		
Large triploid	26.8 ± 0.34	2.0 ± 0.05	74.9 ± 0.60[c]	61.2 ± 0.65[d]	18.2	56.6

[1] Mean per tray, of 50 oysters ± se (n=6). Data were transformed log Y prior to analysis to ensure homogeneity of variance.

[2] Within columns, for final sizes, means with different letters differ significantly. For initial heights and weights, only size category was significant ($P < 0.05$).

[3] $\frac{[(\text{final triploid size-initial triploid size}) - (\text{final diploid size-initial diploid size})]}{\text{final diploid size-initial diploid size}} \times 100$

Table 2 Results of mixed model ANOVA of $\log_e$ transformed whole oyster weights at the end of a 24 months growth comparison of small and large, diploid and triploid Sydney rock oysters, *Saccostrea commercialis*, grown in Port Stephens, New South Wales.

Source of variation	Fixed or random	Sum of squares	df Effect	MS Effect	df[1] Error	MS Error	F	P	Terms in MS
Block (A)	R	0.0020	1	0.0020	0.0323	0.0002	11.934	0.889	AxB + AxC - AxBxC
Ploidy (B)	F	0.8246	1	0.8246	1	0.0006	1453.7	0.0167	AxB
Size (C)	F	0.1665	1	0.1665	1	0.0003	639.8	0.0252	AxC
AxB	R	0.0006	1	0.0006	1	0.0007	0.855	0.5249	AxBxC
AxC	R	0.0003	1	0.0003	1	0.0007	0.392	0.6439	AxBxC
BxC	R	0.0286	1	0.0286	1	0.0007	43.1	0.0962	AxBxC
AxBxC	R	0.0007	1	0.0007	16	0.0013	0.505	0.4874	error
Error		0.0210	16	0.0013					
Total		1.0441	23						

[1]df of error terms computed using the Satterthwaite method

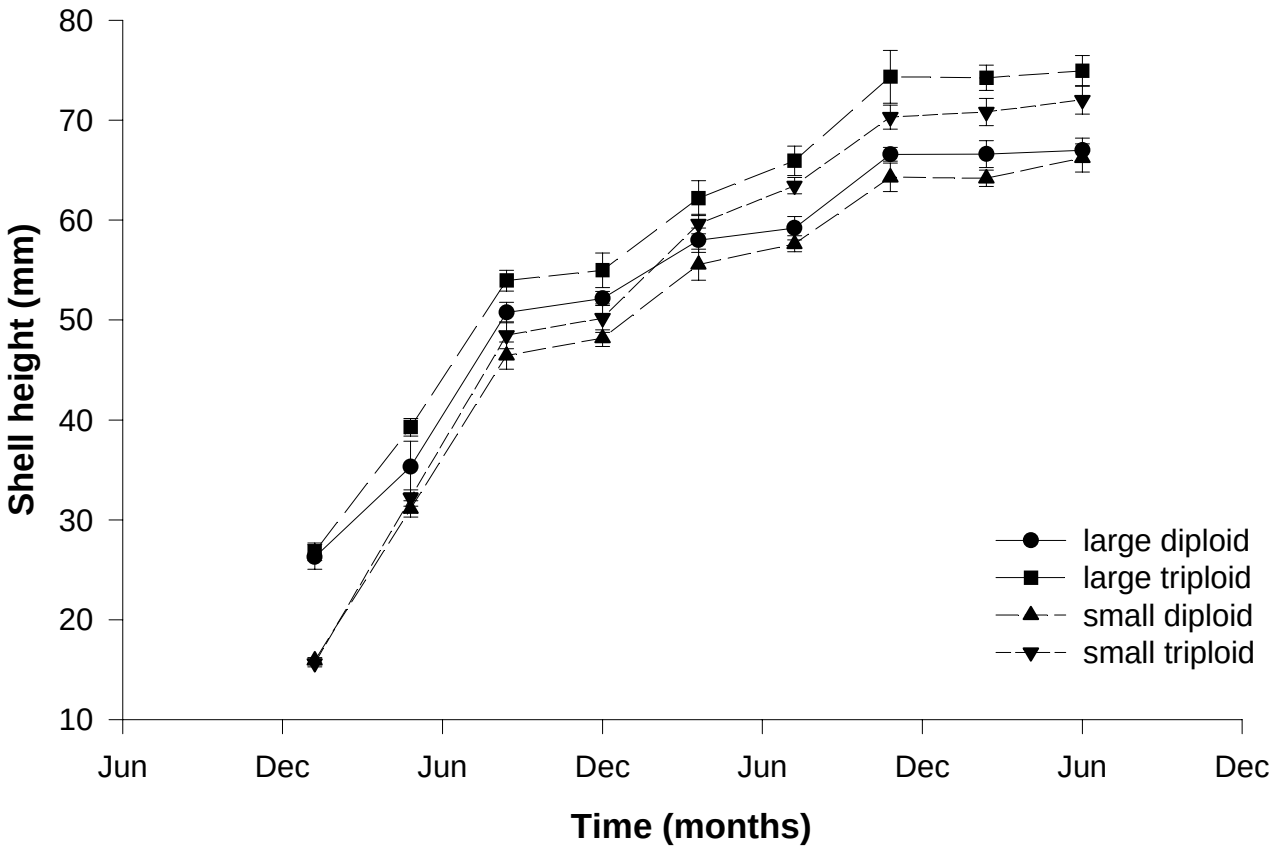

Figure 1 Comparison of shell heights (mm) of two grades of diploid and triploid Sydney rock oysters, *Saccostrea commercialis*, at North Arm Cove, Port Stephens, December 1994 - December 1996 (means ± 95% confidence intervals).

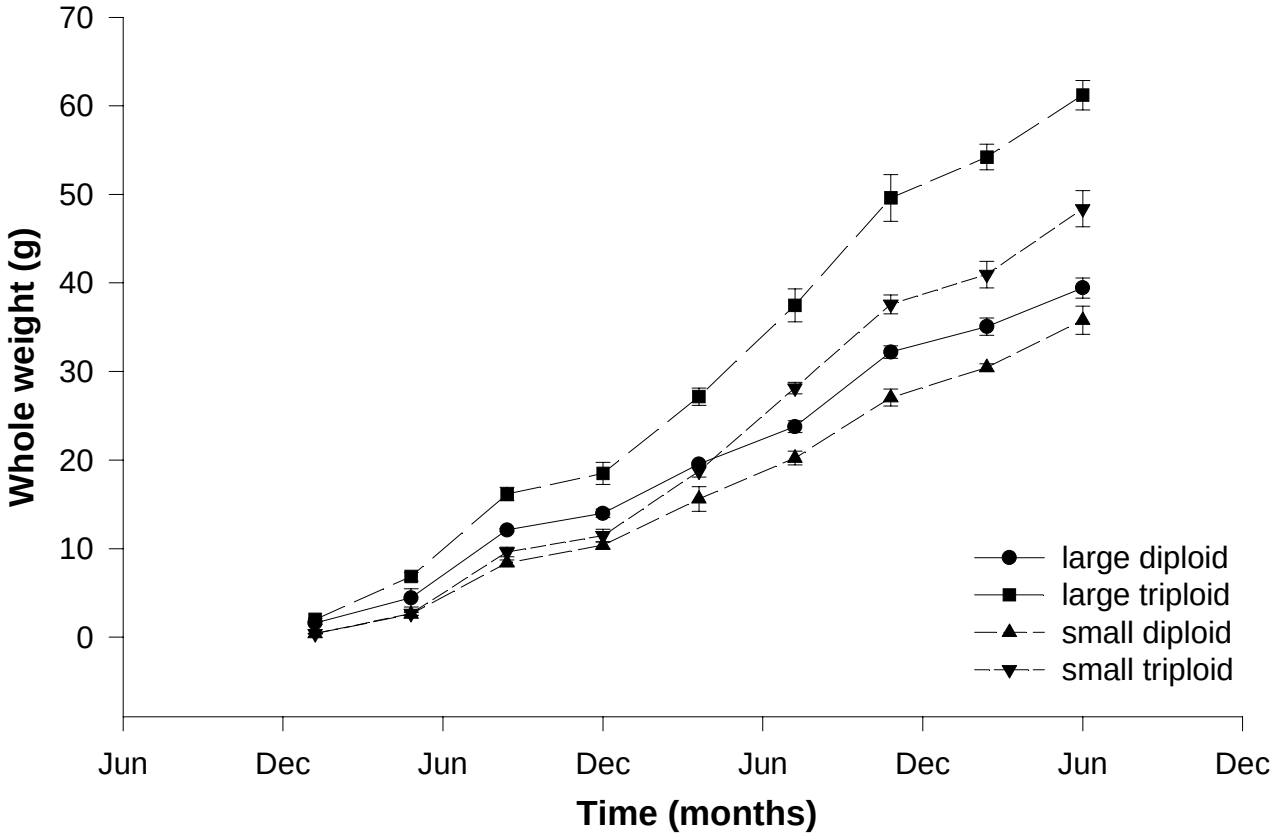

Figure 2 Comparison of whole weight (g) of two grades of diploid and triploid Sydney rock oysters, *Saccostrea commercialis*, at North Arm Cove, Port Stephens, December 1994 - December 1996 (means ± 95% confidence intervals).

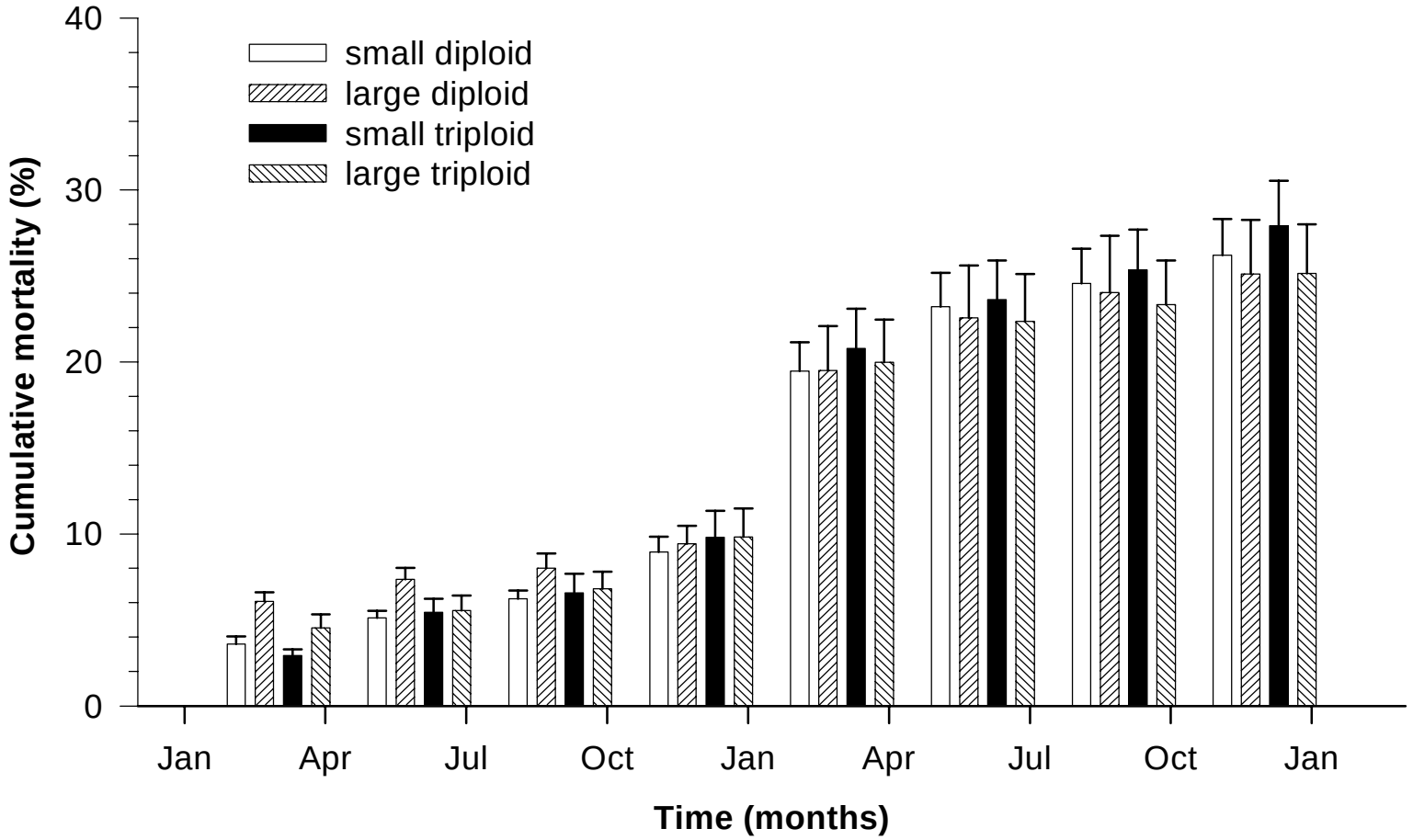

Figure 3 Cumulative mortality of two size grades of diploid and triploid Sydney rock oysters, *Saccostrea commercialis*, grown at North Arm Cove, Port Stephens from December 1994 to December 1996. Means ± s.e.

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STUDIES ON TRIPLOID OYSTERS IN AUSTRALIA. X. GROWTH AND MORTALITY OF DIPLOID AND TRIPLOID SYDNEY ROCK OYSTERS, *SACCOSTREA COMMERCIALIS* (IREDALE AND ROUGHLEY)

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ABSTRACT Growth and mortality of triploid Sydney rock oysters, *Saccostrea commercialis* (Iredale and Roughley) were compared to those of sibling diploids at 13 oyster farms in ten estuaries in New South Wales (NSW). Although results varied between farms, after 2-2½ years on commercial oyster leases triploids were on average 30.7% heavier and 8.6% larger in shell height than sibling diploids. Seven of the 13 farms had triploids with a mean weight of at least market size (40 g) after 2-2½ years while no farms had diploids of the same mean weight. The growth advantage of triploids began to be expressed at specific sizes rather than ages, specifically at a mean whole weight above 5-10 g or shell height of 30-40 mm. In general, triploids appeared to grow faster relative to diploids at higher water temperatures. Although readily accepted by processors during cooler seasons, triploid Sydney rock oysters developed a patchy, discolouration of the gonad during warmer months that may affect marketability during summer. Mortality of triploids was significantly ($p<0.01$) lower than that of diploids at six of the 13 farms and did not differ ($p>0.05$) at six of the remaining seven farms. The growth coefficients were lower and mortality higher for wild-caught diploids than both hatchery reared diploids and triploids. Triploid Sydney rock oysters were found to offer significant commercial advantages over diploid oysters because of faster growth rates and lower mortality when grown under commercial oyster farming conditions across a range of estuaries in NSW.

Short running title: FARMING TRIPLOID *SACCOSTREA COMMERCIALIS*
KEY WORDS: Growth, farming, oysters, triploid, mortality

INTRODUCTION

Sydney rock oysters generally take from 3 to 4 years to reach marketable size (Nell 1993) compared to 1½-3 years for Pacific oysters, *Crassostrea gigas*, Thunberg (Graham 1991) in Australia. Increasingly, Pacific oysters (predominantly from Tasmania and South Australia) are taking over traditional Sydney rock oyster markets. Adoption of triploid technology may help New South Wales' (NSW) farmers to remain competitive against industries based on the faster growing Pacific oyster. Nell et al. (1994) compared the performance of triploid and diploid Sydney rock oysters in a small scale trial in Port Stephens, NSW over two and a half years. At the end of the study, the triploids were on average 41% heavier and reached market size 6-18 months earlier than their diploid siblings. However, results varied among the four sites tested within the one estuary (range 32-48.8% difference in whole weights of diploids and triploids). The benefits of triploidy are known to be influenced by environmental factors such as food availability, (Davis 1989a) and temperature (Davis 1989b, Shpigel et al. 1992). Oyster farming in NSW is practised throughout the coastal areas of the state, across a range of environmental conditions (particularly temperature). At the conclusion of the preliminary study, there were no commercial bivalve hatcheries operating in NSW. To commercialise production and farming of triploid Sydney rock oysters, potential hatchery operators needed to be assured that the benefits of triploidy could be extended to other oyster growing estuaries, using commercial farming methods. In this study, to enable commercialisation of triploid technology, we compared the growth and mortality of diploid and triploid Sydney rock oysters grown

on oyster farms throughout NSW by commercial oyster farmers. This study also provides a comparison of normal, diploid oyster growth across a range of NSW estuaries.

METHODS

Induction of Triploidy and Ploidy Determination

Triploid (300 x 10^6) and diploid (60 x 10^6) sibling larvae were produced in February 1994 from 48 female and 6 male oysters from Port Stephens, NSW. Triploidy was induced in zygotes using the method described in Nell et al. (1996) with a cytochalasin B (CB) concentration of 1.25 mg/L. Larvae were stocked directly into 20 000 l larval rearing tanks (3 triploid and 1 diploid).

Percentage triploidy was determined by direct chromosome counts at 4 h in embryos (Nell et al. 1996) and by flow cytometry for shelled larvae and spat.

Larvae and Spat Rearing

Larvae were reared using standard hatchery techniques for Sydney rock oysters (Frankish et al. 1991). The 3 triploid treatment tanks were combined by day 5 due to low survival and similar percent triploid readings (78.3-81.5%). Screening at water changes was conservative to allow for initial slow growth of triploid larvae following exposure to CB (Wada et al. 1989). Larvae were settled on ground scallop shell between days 18-22 and reared in the hatchery for 5 weeks before being transferred to outdoor upwelling units. Spat were on-grown to a size of 7-10 mm.

Oyster Management

Thirteen commercial oyster farmers in eleven different estuaries throughout NSW (Fig. 1) from Pambula Lake (36º 58´S, 149º 54´E) to Hastings River (31º 25´S, 152º 55´E) were each sold 25 000 diploid and 25 000 sibling triploid spat between July and November 1994. Rather than supplying oysters matched for initial size (ie which would risk matching fast diploids with slow triploids), oysters were supplied to farmers as they reached a growout size of 7-10 mm (shell height). Mean whole weight of spat supplied to farmers ranged from 0.07±0.02 to 0.11±0.05 g and 0.07±0.02 to 0.10±0.03 g (mean±sd; n=400) for diploids and triploids, respectively. Mean shell height ranged from 8.40±0.82 to 9.85±1.68 mm and 8.22±0.83 to 9.73±1.05 mm (mean±sd; n=400) for diploids and triploids, respectively. All farmers were encouraged to use standardised stocking densities, throughout the experiment, of 2 L for cylinders and 50% coverage for tray culture (Nell 1993). However, in the colder South coast areas ie Lake Pambula and Lake Merimbula, where conditions produce limited oyster growth in cylinders at these densities, they were reduced. Baskets (Maguire et al. 1994a) were stocked at between 1-2 L. Table 1 shows a summary of culture methods used at each farm. Oysters were grown by the farmers using standard commercial oyster farming techniques and culture equipment was provided by farmers. For this reason culture trays, baskets, etc. could not be standardised across all estuaries, however, diploids and triploids at each farm were grown under the same conditions. The normal farming practice of grading oysters into several size grades, when there is a large range of sizes to allow growth of the smaller oysters without competition from the larger grades, was minimised where possible to make comparisons between ploidy levels and farms simpler. However, as this was a study based on commercial farming methods and large size ranges developed at least at some stage during the course of the experiment (at all sites except for Tilligerry Creek, Port Stephens (farm 1) and Woolooware Bay) oysters were graded when necessary. Grades were recombined when there was no longer a difference in size.

Oysters from Georges River (farm 2) and Lake Pambula were divided among three sites when they reached a large enough volume (after 2 and 6 months respectively). Oysters from Georges River (farm 2) were divided between on upriver lease and two downriver leases. During the first year, oysters from the upriver site suffered high mortality with symptoms consistent with the disease QX. After 12 months this site was no longer included in the study due to very poor survival and the remaining two downriver sites were combined. QX disease, which is caused by the protistan parasite *Marteilia sydneyi*, spread to the Georges River in 1994 (Adlard and Ernst 1995).

As is commercial practice, oysters located in estuaries subject to the disease winter mortality, were moved where possible (Tilligerry Creek: farms 1 and 2; Georges River: farms 1 and 2;

Woolooware Bay and Clyde River) to a lease further upstream or to a higher rack (Lake Merimbula) during winter. Winter mortality is caused by the protistan parasite *Mikrocytos roughleyi* (Farley et al. 1988).

A further comparison of a cylinder of "wild-caught" single-seed (Nell 1993) diploid oysters to the hatchery produced diploids and triploids was set up at Georges River (farm 1), Lake Merimbula and Brisbane Waters.

Sampling Strategy

A sub sample of 400 of each type of oyster was measured for whole weight and shell height before being supplied to farmers then every 2 months thereafter at each farm until November 1995 when the sampling frequency was changed to every 3 months. Where several grades were present a smaller sample was measured for each grade ie 400-600 total per ploidy type and a weighted mean determined. Samples for individual height and weight measurements were taken from a random 10% of stock brought in every 2-3 months. For this reason mortalities were not removed during the course of the experiment; ie to maintain similar densities of oyster shells and percentage dead between sampled trays and trays remaining on leases. Mortality was assessed from a sub sample and returned to trays for a final count at the end of the experiment.

Total weight of stock was measured every 2 months for the first 6-9 months to assess losses and the proportion in each grade. As handling became more difficult with the large increase in volume during the first year (after March 1995), farmers were given the choice of bringing in either 25% of stock or everything every 6 months to find the total weight of all oysters. Weights of dead oysters and overcatch were accounted for when total weights were measured.

Salinity and temperature data were measured weekly by farmers using a thermometer and hydrometer.

Statistical Analyses

All whole oyster weight and shell height data are presented in figures as means±95% confidence intervals. Weighted means of whole weight, shell height and mortality data were calculated for farms with more than one size grade or site:

$$\overline{X}_w = \frac{\sum^{n} W_i \overline{X}_i}{\sum^{n} W_i}$$

where X_W is the weighted mean calculated from the mean, $\overline{X}_i$ of each of n grades, and each $\overline{X}_i$ weighted by a factor W_i (Sokal and Rohlf 1995). The total number in each grade was used as a weighting factor and was estimated from the total weight of all oysters for each grade divided by the mean weight of individual oysters for that grade.

Growth coefficients (G_{90}) were calculated for comparisons of hatchery stock with wild-caught diploids to account for differences in initial whole weights and in the duration of the experiment (Spencer and Gough 1978):

$$G_{90} = \frac{90}{\text{Duration (days)}} \times \ln\left(\frac{\text{Final weight (g)}}{\text{Initial weight (g)}}\right)$$

Mortality data were analysed for significant differences (p = 0.05) by Chi-squared contingency tables (Sokal and Rohlf 1995). Initial and final height and weight data were analysed for significant differences using ANOVA (Sokal and Rohlf 1995) after homogeneity of variance was confirmed using Cochran's test (Winer 1991). Size data were $\log_{10}$ transformed where necessary.

Where several grades were present at the end of the experiment a random subsample (the size of which was determined by the proportions in each grade) was used for ANOVA.

RESULTS

Induction of Triploidy

The mean triploidy reading on day 0 was 79.9±0.92% (mean±se; n=3 groups of 60 larvae) which increased to 87.8% by 10 weeks' post-settlement. Flow cytometry of biopsied gill tissue from oysters from three farms (Hastings River, Karuah River and Clyde River) in January/February 1996 gave a mean triploid level of 88±4.0% (mean±se, n=3 groups of 183-292 oysters).

Larval and Spat Rearing

Poor water quality during the larval run resulted in low survival to settlement (7.5% diploids and 2.0% triploids) with set rates of 56.3% for diploids and 43.2% for triploids. Spat in upweller systems were affected by "post-settlement mortality" (Frankish et al. 1991) in April 1994. Survival from settlement in early March 1994 to May 1994 was 26% for diploid and 31% for triploid spat, respectively. As spat were graded over the same mesh but not matched for size before being supplied to farmers (to avoid matching slow growing triploids with fast diploids) initial sizes of diploids and triploids were sometimes different. However, this was generally in favour of the diploids with initially larger diploids ($p<0.05$) at eight of the 13 farms, larger triploids at two farms (Hastings River and Tilligerry Creek, 1) and no significant difference ($p > 0.05$) between the initial size of diploids and triploids at Tilligerry Creek, 2, Brisbane Waters and Shoalhaven.

Appearance

A brown to grey colour appearing as distinct patches on the gonad were noted on triploid oysters. This colouration developed during the second year on leases and was most noticeable during summer months.

Whole Oyster Weight

Whole oyster weights over the 2½ year study are shown in Figure 2. For all farms except Lake Merimbula, mean triploid whole weight was greater than mean diploid weight from the time when oysters reached a whole weight of about 5-10 g. Triploids generally remained heavier until the end of the study. Average time taken to reach a mean whole weight of 10 g was 13 months for triploids and 14 months for diploids (Table 2). Largest increases in mean whole oyster weight occurred during spring through to autumn for northern and central NSW estuaries (Hastings down to Shoalhaven Rivers). The growth season for oysters on the south coast occurred later, ie summer through to autumn/early winter. Greatest relative growth of triploids compared to diploids generally occurred later in the growth season after the first year on leases. By the end of the 2½ year study the mean whole oyster weight from the 13 farms involved in the study was 28.3 g (range 18.7-37.4 g) for diploids and 37.0 (20.9-49.4 g) for triploids, a difference of 30.7% (Table 3). Final weights of triploids were significantly greater ($p<0.01$) than diploid weights at all 13 farms with an apparent effect of temperature (Fig. 4) on the relative growth of diploids and triploids. The seven sites with the greatest difference between diploid and triploid growth had mean water temperatures of 18ºC or more, whilst four of the six remaining sites had mean water temperatures of less than 18ºC. Time to market size could not be compared between the two ploidy types as diploids had not reached this size by the end of the study, instead the time to reach a mean whole weight of 30 g was compared. Where data are available for both ploidy groups, triploids reached this size 20% faster than diploids. The average time taken for triploids to reach a mean whole weight of 40 g was 26 months for the seven farms that had oysters at this size. There were no farms with diploids at a mean weight of 40 g.

Shell Height

Shell growth of diploids and triploids followed a similar pattern to whole weight (Figure 3). In general, by the time oysters had reached a mean shell height of 30 to 40 mm, growth of triploids

was greater than that of diploids. Triploids at most farms had reached a shell height of approximately 40 mm, after 8-12 months on leases. Fastest shell growth occurred between winter/spring and autumn for northern and central sites and between spring/summer and autumn for sites south of the Shoalhaven River. Shell growth of triploids relative to diploids was greatest during spring and summer for northern and central sites and between spring and autumn for southern sites. After 2-2½ years on oyster leases mean shell height was 61.1 mm for diploids and 66.4 mm for triploids, a difference of 8.6% (Table 3) and was significantly larger ($p<0.001$) for triploids at 12 of the 13 farms.

Cumulative Mortality

There was little difference between cumulative mortality of diploids and triploids at most sites during the first 12-18 months on leases (Figure 5). However, cumulative mortality (Table 4) of triploids at the end of the study was significantly ($p<0.01$) lower than that of diploids at six of the 13 farms and did not differ ($p > 0.05$) at six of the remaining seven farms. Cumulative mortality was higher for triploids than diploids at only one site (Tilligerry Creek, farm 1; $p < 0.05$).

Wild-Caught Diploid: Hatchery Diploid and Triploid Comparison

Initial whole oyster weights and shell heights (Tables 5 and 6) of wild-caught diploids supplied by oyster farmers were significantly ($p < 0.05$) different from those of hatchery stock for all three sites (data were not homogeneous for the Georges River and Brisbane Waters sites). There was also a significant difference ($p < 0.05$) between hatchery diploids and triploids at the Georges River site (for both height and weight) and Lake Merimbula (weights). For this reason growth was compared using growth coefficients (Table 7). Wild-caught diploids had lower growth coefficients than both diploid and triploid hatchery stock at all three sites, except for hatchery diploids at Lake Merimbula. High mortality (with symptoms consistent with those of the disease winter mortality) of wild-caught diploids occurred at the Brisbane Waters site during the first year resulting in insufficient numbers to continue the comparison. The cumulative mortality of wild-caught oysters after only 11 months was 55.4% compared to only 6.0 and 2.7% for hatchery diploids and triploids respectively. For Georges River and Brisbane Waters sites there was a significant effect ($p < 0.05$) of ploidy type on cumulative mortality (Table 7).

DISCUSSION

The variation in performance of triploid Sydney rock oysters between different sites in the preliminary study of Nell et al. (1994) was emphasised in the present commercial-scale study. Much of the variation in this study may be attributable to differences in water temperatures. However, growth of both diploids and triploids at Brisbane Waters, Lake Merimbula and Hawkesbury River appears to have been influenced by an additional factor, possibly food availability (L. Cooper pers. comm.). Davis (1989b) also measured faster growth rates of triploids relative to diploids at a site with maximum water temperatures (July and August) of 20ºC compared with a site with maximum water temperatures of 16ºC. This is most likely due to the relatively greater contribution by diploids of energy reserves to gametogenesis at higher temperatures (Shpigel et al. 1992). The unusually large difference (98.9%) between diploids and triploids at the Hastings River site is partly due to the poor growth of diploids. Oysters at this site were grown subtidally which may account for the faster growth of triploids at this site (Nell et al. 1994). However, growth of diploids appeared to be retarded even before they reached a size at which we would expect gametogenesis/spawning to affect their growth (Figure 2).

The growth seasons for both diploids and triploids were consistent with previous studies (Allen and Downing 1986, Nell et al. 1994), ie spring through to autumn with greater relative growth of triploids compared to diploids later in the growth season. The period of greater relative growth of triploids occurred prior to the normal spawning season (February to May) for Sydney rock oysters (Nell 1993), ie when diploids are diverting a large proportion of their energy stores to gametogenesis at the expense of somatic growth. Triploid Sydney rock oysters did not show an advantage over diploids until they reached a mean whole weight of between 5 to 10 g or shell height of 30 to 40 mm.

This corresponded to a growout time of between 8 and 14 months and is similar to results (12-13 months) obtained for Pacific oysters in Japan (Akashige and Fushimi 1992) and earlier results (6-9 months) for Sydney rock oysters (Nell et al. 1994). The large time range for the 13 farms emphasises the fact that benefits of triploids relative to diploids are dependent on size rather than age. This is because the onset of gametogenesis and the ability to spawn in diploid oysters is related to oyster size rather than age. Singh (1978) found that the American oyster, *Crassostrea virginica* Gmelin, is capable of spawning when it reaches a size of around 25 mm. This is a similar to the size (30-40 mm) at which the slower relative growth of Sydney rock oyster diploids compared to triploids became apparent in this study.

After 2½ years on commercial oyster leases, triploid Sydney rock oysters were 30.7% heavier (whole oyster weight) and 8.6% larger (shell height) than sibling diploid oysters. In addition, the generally lower growth coefficients of wild-caught diploids compared with hatchery stock at three sites are contrary to the belief of many commercial farmers that hatchery stock is slower growing than wild-caught seed. However, the data for Georges River and Lake Merimbula should be interpreted with caution as wild-caught seed may have been slower growers within a cohort, ie they were provided by farmers out of a small size grade from a larger batch of oysters.

There are numerous and conflicting reports on the relative survival of diploids and triploids. The majority suggest similar survival of diploids and triploids post-metamorphosis (eg Stanley et al. 1981, Chaiton and Allen 1985, Nell et al. 1994). Davis (1989a) found that, under starvation conditions, diploids survived better than triploids. In contrast, triploid American oysters have been shown to be less susceptible than diploids to the disease MSX, caused by the parasite *Haplosporidium nelsoni* (Matthiessen and Davis 1992) and our present results with Sydney rock oysters illustrate a clear trend of greater triploid survival compared to sibling diploids. This was particularly noticeable at sites affected by the disease winter mortality. Both ploidy types are susceptible to winter mortality (Nell et al. 1994); however, despite identification of winter mortality symptoms (*eg.* lesions on the gills and labial palps) fewer triploids died from the disease (Hand et al. 1998). In contrast, Nell et al. (1994) found no significant difference between mortality of diploids and triploids when grown in Woolooware Bay, NSW where oysters are prone to winter mortality.

Discolouration of triploid oysters has only been reported once previously when 5.9% of triploid Pacific oysters developed brown discolouration during summer (Maguire et al. 1994b). In Sydney rock oysters, discolouration did not appear to affect the oyster in any other way and was less obvious during the colder months when triploids were readily accepted by oyster processors. As this will most likely be the time when triploids are more marketable, due to their better meat condition compared to diploids (Nell et al., 1994), it is unlikely to affect their commercialisation.

Sydney rock oysters currently take from 3-4 years to reach market size (Nell 1993) and may take longer on the colder south coast of NSW. Seven of the 13 farms in this study had marketable triploid Sydney rock oysters (over 40 g mean whole weight) after 2-2½ years on leases whilst there were no farms with diploid oysters at this size. Final mean whole oyster weight of triploids was significantly greater ($p < 0.01$) than diploid weight at all 13 farms. Labour is one of the major costs of oyster production from spat (Graham 1991). This would be reduced significantly by a reduction in growout time of at least 20%. The better survival of triploid hatchery stock compared to wild-caught diploids and better meat condition during winter compared to diploids would further improve the profitability of farming triploid oysters.

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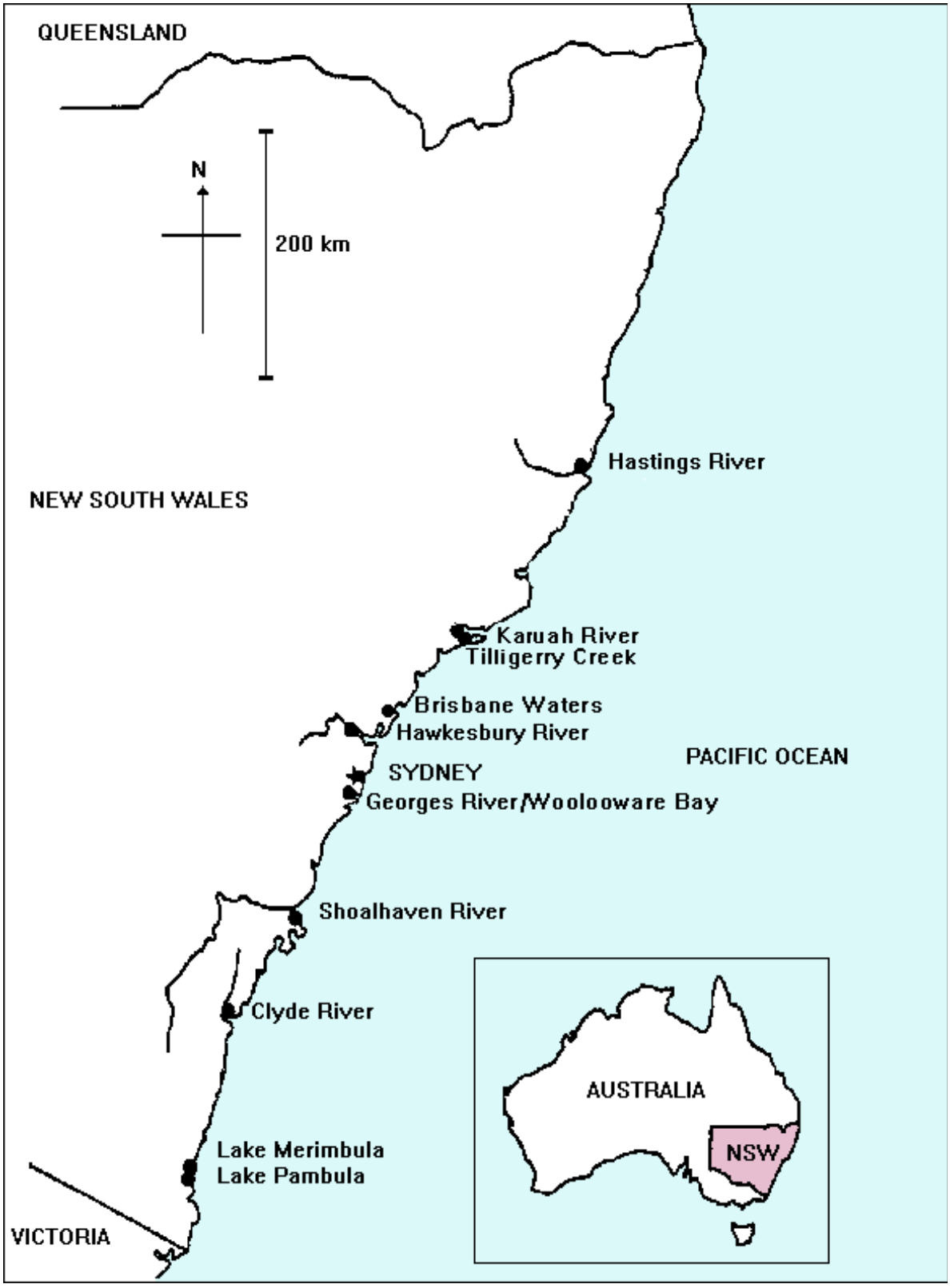

Figure 1. Estuaries in NSW where farming sites were located (adapted from Holliday et al. 1988).

Table 1. Culture methods for diploid and triploid Sydney rock oysters *Saccostrea commercialis* grown by commercial oyster farmers in New South Wales from July 1994 to November/December 1996.

Estuary	Month Supplied (1994)	Nursery Culture		Growout	
		Culture method	Stocking density	Culture method	Management
Hastings River	July	Subtidal trays	50% coverage	Subtidal trays	Oysters dried out 1-7 days every 4 weeks
Tilligerry Creek (farm 1)	"	Intertidal trays	50% coverage	Intertidal trays	Moved during winter
Tilligerry Creek (farm 2)	August	Intertidal trays	50% coverage	Intertidal trays	Moved during winter
Karuah River	September	Cylinders	2 l	Baskets Intertidal trays	
Georges River (farm 1)	"	Cylinders	2 l	Baskets Intertidal trays	Moved during winter
Georges River (farm 2)	"	Cylinders	2 l	Baskets Intertidal trays	Moved during winter Divided between 3 sites
Hawkesbury River	October	Cylinders	2 l	Intertidal trays	
Woolooware Bay	"	Intertidal trays	50% coverage	Intertidal trays	Moved during winter
Clyde River	"	Intertidal trays	50% coverage	Intertidal trays	Moved during winter
Lake Merimbula	"	Cylinders	1-2 l	Intertidal trays	Raised during winter
Lake Pambula	"	Cylinders Trays	0.5-2 l 50% coverage	Intertidal trays	Divided between 3 sites
Brisbane Waters	November	Cylinders	2 l	Baskets Intertidal trays Large cylinders[a]	Large grades moved between large cylinders and trays
Shoalhaven River	"	Cylinders	0.5-2 l	Intertidal trays Subtidal trays	After 9 months on intertidal trays Transferred to subtidal culture for 6 months

[a]200 l rotating cylinders with 12 mm mesh, similar to the smaller Stanway® cylinders.

Table 2. Time[a] taken (months) to reach a whole weight of 10, 20, 30 and 40 g by diploid and triploid Sydney rock oysters *Saccostrea commercialis* grown by commercial oyster farmers in New South Wales.

Estuary	Site	10 g		20 g		30 g		40 g	
		Diploid	Triploid	Diploid	Triploid	Diploid	Triploid	Diploid	Triploid
Hastings River[b,c]		15	12	28	16	-	22	-	27
Tilligerry Creek (farm 1)		12	11	18	17	23	20	-	24
Tilligerry Creek (farm 2)		14	13	20	17	26	22	-	26
Karuah River		11	8	15	14	23	19	-	26
Georges River (farm 1)		14	11	18	16	25	19	-	25
Georges River (farm 2)	1	12	11	17	16	26	18	-	25
	2	8	7	17	16	26	18	-	25
Hawkesbury River		14	15	19	18	-	27	-	-
Woolooware Bay		14	15	19	18	-	25	-	-
Clyde River[b]		14	13	17	14	26	19	-	26
Lake Merimbula[b]		16	17	-	26	-	-	-	-
Lake Pambula[b]	1	17	16	25	20	-	-	-	-
	2	16	15	27	23	-	-	-	-
	3	15	15	20	19	-	-	-	-
Brisbane Waters[b]		15	16	22	20	-	24	-	-
Shoalhaven River		12	11	16	14	-	22	-	-
Average time[d]		14	13	(19)	17	(25)	(20)	-	(26)

[a]Actual time taken, estimated from graph of whole weight against time; - represents oysters that have not reached set size.
[b]A weighted mean of more than 1 size grade was calculated.
[c]Subtidal culture.
[d]Mean time for all farms; Months in brackets do not include data from slower growing sites where times are not available for that set size and for both ploidy types

Table 3. Whole weight and shell height of diploid and triploid Sydney rock oysters *Saccostrea commercialis* grown by commercial oyster farmers in New South Wales from July 1994 to November/December 1996.

Estuary	Month Supplied (1994)	Whole Oyster Weight (g)		Difference (%)[a]	Shell Height (mm)		Difference (%)[2]
		Diploid	Triploid		Diploid	Triploid	
Hastings River[b,c]	July	24.9 ± 0.5	49.4 ± 0.8	98.9	60.4 ± 0.5	69.6 ± 0.5	15.2
Tilligerry Creek (farm 1)	"	34.4 ± 0.3	44.8 ± 0.5	30.1	68.0 ± 0.3	74.0 ± 0.4	8.9
Tilligerry Creek (farm 2)	August	33.0 ± 0.3	44.8 ± 0.5	35.8	69.6 ± 0.3	75.1 ± 0.4	7.9
Karuah River	September	37.4 ± 0.4	47.8 ± 0.5	28.0	66.1 ± 0.3	69.8 ± 0.3	5.5
Georges River (farm 1)	"	33.5 ± 1.1	42.6 ± 0.5	27.1	60.7 ± 0.8	66.6 ± 0.3	9.7
Georges River (farm 2)	"	30.3 ± 0.3	43.9 ± 0.5	44.9	60.5 ± 0.3	68.1 ± 0.4	12.5
Hawkesbury River	October	19.6 ± 0.2	24.6 ± 0.3	25.3	52.2 ± 0.3	59.0 ± 0.4	13.1
Woolooware Bay	"	28.7 ± 0.3	32.3 ± 0.4	12.6	58.8 ± 0.3	62.4 ± 0.3	6.0
Clyde River[b]	"	30.3 ± 0.4	39.9 ± 0.6	32.0	63.3 ± 0.4	68.7 ± 0.5	8.6
Lake Merimbula[b]	"	18.7 ± 0.3	20.9 ± 0.4	11.8	51.7 ± 0.4	56.6 ± 0.4	9.5
Lake Pambula[b,d]	"	21.9 ± 0.5	26.9 ± 0.6	23.0	57.0 ± 0.6	62.2 ± 0.6	8.4
Brisbane Water[b]	November	26.7 ± 0.5	28.2 ± 0.7	5.6	63.3 ± 0.5	63.7 ± 0.7	0.8
Shoalhaven River	"	28.8 ± 0.3	35.3 ± 0.5	22.6	63.3 ± 0.3	66.9 ± 0.4	5.6
Average		28.3	37.0	30.7	61.1	66.4	8.6

[a]Difference (%) = (triploid - diploid)/diploid x 100.
[b]A weighted mean ± se of more than 1 size grade was calculated for mean whole weight and shell height.
[c]Subtidal culture.
[d]Oysters divided between three sites.

Table 4. Comparison[a] of cumulative mortality of diploid and triploid Sydney rock oysters *Saccostrea commercialis* grown by commercial oyster farmers in New South Wales from July 1994 to November/December 1996.

Estuary	Month Supplied (1994)	Cumulative Mortality (%)		Mean Annual Temperature (°C)
		Diploid	Triploid	
Hastings River[b]	July	32.86	26.22**	19.1
Tilligerry Creek (farm 1)	"	11.06	14.62*	20.4
Tilligerry Creek (farm 2)	August	17.15	16.83	19.7
Karuah River	September	4.80	4.16	20.2
Georges River (farm 1)	"	48.45	24.31***	18.1
Georges River (farm 2)[c]	"	61.11	24.12***	18.0
Hawkesbury River	October	5.26	3.71	19.6
Woolooware Bay	"	25.73	20.48**	17.6
Clyde River[b]	"	18.89	15.76	18.3
Lake Merimbula[b]	"	9.15	10.31	18.0
Lake Pambula[b,d]	"	28.60	13.10***	16.7
Brisbane Water[b]	November	94.61	27.66***	18.8
Shoalhaven River	"	7.70	7.09	17.8

[1]*** = $p<0.001$; ** = $p<0.01$; * = $p<0.05$.
[2]A weighted mean of more than 1 size grade was calculated for cumulative mortality.
[3]Excludes oysters from upriver lease that were removed from the experiment in September 1995 due to low numbers.
[4]Oysters divided between three leases.

Table 5. Initial and final[a] whole weights[b] of diploid, triploid and wild caught diploid Sydney rock oysters *Saccostrea commercialis* grown by commercial oyster farmers in New South Wales.

Estuary	Initial Whole Oyster Weight (g)			Final Whole Oyster Weight (g)		
	Diploid	Triploid	Wild caught diploid	Diploid	Triploid	Wild caught diploid
Georges River[c]	0.4 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	24.9 ± 0.3	33.8 ± 0.4	30.4 ± 0.5
Lake Merimbula	1.4 ± 0.0	1.1 ± 0.0	1.3 ± 0.0	24.5 ± 0.3	26.4 ± 0.4	24.6 ± 0.3
Brisbane Waters[c]	0.1 ± 0.0	0.1 ± 0.0	0.8 ± 0.0	3.3 ± 0.1	2.0 ± 0.1	9.9 ± 0.5

[a]Georges River: November, 1994-May, 1996, Lake Merimbula: April, 1995-December, 1996, Brisbane Waters: November, 1994-September, 1995.
[b]Mean±se (n=200), a weighted mean of more than 1 size grade was calculated for Brisbane Waters.
[c]Comparison was terminated after only 11 months due to high mortality and insufficient numbers of wild caught diploids.

Table 6. Initial and final[a] shell heights[b] of diploid triploid and wild caught diploid Sydney rock oysters *Saccostrea commercialis* grown by commercial oyster farmers in New South Wales.

Estuary	Initial Shell Height (mm)			Final Shell Height (mm)		
	Diploid	Triploid	Wild caught diploid	Diploid	Triploid	Wild caught diploid
Georges River[c]	15.5 ± 0.1	16.1 ± 0.1	17.9 ± 0.2	61.0 ± 0.3	65.7 ± 0.4	63.8 ± 0.6
Lake Merimbula	24.0 ± 0.3	23.3 ± 0.3	22.5 ± 0.3	56.6 ± 0.4	60.4 ± 0.4	59.2 ± 0.4
Brisbane Waters[c]	9.8 ± 0.1	9.7 ± 0.1	21.5 ± 0.5	25.0 ± 0.4	22.1 ± 0.4	40.9 ± 0.9

[a]Georges River: November, 1994-May, 1996, Lake Merimbula: April, 1995-December, 1996, Brisbane Waters: November, 1994-September, 1995.
[b]Mean±se (n=200), a weighted mean of more than one size grade was calculated for Brisbane Waters.
[c]Comparison was terminated early due to high mortality and insufficient numbers of wild caught diploids.

Table 7. Growth coefficients[a] and cumulative mortality of diploid, triploid and wild caught diploid Sydney rock oysters *Saccostrea commercialis* grown by commercial oyster farmers in New South Wales.

Estuary[b]	Growth coefficient (G_{90})			Cumulative mortality (%)		
	Diploid	Triploid	Wild caught diploid	Diploid	Triploid	Wild caught diploid
Georges River[c]	0.676	0.726	0.617	8.5	6.2	48.2
Lake Merimbula	0.420	0.466	0.431	10.1	8.5	11.6
Brisbane Waters[c,d]	1.016	0.899	0.755	6.0	2.7	55.4

[a] $G_{90} = \frac{90}{Duration\ (days)} \times \ln\left(\frac{W_t}{W_0}\right)$

Where: W_t = Final mean weight of oysters and W_o = Initial mean weight of oysters.

[b] Georges River: November, 1994-May, 1996, Lake Merimbula: April, 1995-December, 1996, Brisbane Waters: November, 1994-September, 1995.

[c] Comparison was terminated early due to high mortality and insufficient numbers of wild caught diploids.

[d] A weighted mean of more than one size grade was used for calculation of the Growth Coefficient.

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STUDIES ON TRIPLOID OYSTERS IN AUSTRALIA. XI. SURVIVAL OF DIPLOID AND TRIPLOID SYDNEY ROCK OYSTERS (*SACCOSTREA COMMERCIALIS* (IREDALE AND ROUGHLEY)) THROUGH OUTBREAKS OF WINTER MORTALITY CAUSED BY *MIKROCYTOS ROUGHLEYI* INFESTATION

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ABSTRACT Diploid and triploid Sydney rock oysters, *Saccostrea commercialis,* were grown at seven sites in New South Wales (NSW) for 25 to 28 months and exposed to the parasite *Mikrocytos roughleyi* over two consecutive winters during the period August-November 1994 to December 1996. Triploids showed a higher survival than diploids. Over the second winter/spring average cumulative mortality of diploids across all sites, was 35.0% (range 6.7-76.8%) compared to only 12.2% (range 4.0-18.1%) for triploids. This reduction in mortality during the second year of culture on leases, combined with the growth and condition advantages that triploidy confers, could provide the Sydney rock oyster industry in NSW with significant improvements in profitability.

Short running title: SURVIVAL OF TRIPLOID OYSTERS
***KEY WORDS*:** Disease, oysters, triploid, winter mortality

INTRODUCTION

Production by the oyster farming industry in New South Wales (NSW), Australia is irregularly affected by three principal diseases: winter mortality caused by the protistan parasite, *Mikrocytos roughleyi* (Farley et al. 1988), QX caused by the haplosporidian parasite, *Marteilia sydneyi* (Perkins and Wolf 1976) and mudworms, the most harmful of which is *Polydora websteri* (Skeel 1979).

Winter mortality can cause losses of up to 80% of a crop of market size oysters (Wolf 1967) with most mortality occurring from late winter to spring. The disease is limited to the mid to southern part of the range in which *S. commercialis* is farmed (Nell 1993), corresponding to its greater prevalence in colder waters (Roughley 1926). In addition, cold autumn/winter temperatures following dry autumn weather (resulting in high estuarine salinities) are generally associated with an increase in the severity of infestation by *M. roughleyi* (Wolf 1967). This may account for the frequently observed association between oysters with high meat condition and those affected by winter mortality as spawning is generally stimulated in *S. commercialis* by high temperatures and a reduction in salinity (Nell and Smith 1988). Alternatively, meat condition may have a more direct effect on the incidence of winter mortality.

M. roughleyi infestation of oysters (winter mortality) is characterised by the appearance of yellow to brown lesions on the labial palps, mantle, gills, gonad and/or adductor muscle (Roughley 1926, Wolf 1967). Oysters eventually lose the ability to remain closed when exposed to air at low tide. Despite the identification of the species responsible for winter mortality (Farley et al. 1988), the mechanisms by which *M. roughleyi* infests Sydney rock oysters and its physiological effects are still poorly understood.

Mortality has frequently been compared between diploid and triploid bivalves with varying results (eg. Barber and Mann 1991, Meyers et al. 1991, Mathiessen and Davis 1992). Several authors have proposed that any enhanced performance of triploids over diploid oysters results from 'hybrid vigour'

derived from their greater heterozygosity (eg. Beaumont and Fairbrother 1991, Hawkins et al. 1994). However these authors were referring to meiosis I triploids which are generally (depending on the recombination rate) more heterozygous than both diploids and meiosis II triploids (Guo et al. 1992b, Hawkins et al. 1994). In fact, a comparison of mortalities between meiosis I and meiosis II triploids, and that of diploid oysters frequently illustrates the opposite trend, ie higher mortality of meiosis I triploid bivalves as embryos or during growout compared with both meiosis II triploids and diploids (Stanley et al. 1984, Beaumont and Kelly 1989, Guo et al. 1992a). Consequently the greater heterozygosity of meiosis II triploids compared to diploids would not be expected to influence their relative mortality rates.

In an earlier study in Woolooware Bay, NSW (Nell et al. 1994), a comparison of mature diploid and triploid Sydney rock oysters showed no significant difference in mortality (45.9% of triploids and 41.3% of diploids) over a winter/spring season (May-December 1992). Oysters in Woolooware Bay are frequently affected by winter mortality and the experimental oysters displayed signs typical of the disease. On the basis of that experiment, a difference in susceptibility of diploids and triploids to winter mortality would not be expected. A commercial farming trial of diploids and triploids conducted throughout NSW at 13 oyster farms between 1994 and 1996 revealed, however, a greater overall rate of mortality of diploids compared to triploids (Hand et al. 1998). Data for sibling diploid and triploid oysters are presented here from the seven commercial oyster leases in four NSW estuaries where winter mortality was observed during this study.

METHODS

Production of Spat

The methods of triploidy induction along with those for rearing the larvae and spat are described in the associated commercial farming study on triploid and diploid Sydney rock oysters in NSW (Hand et al. 1998).

Oyster Management

Oysters used in this study formed part of a larger commercial scale farming trial with diploid and triploid Sydney rock oysters on 13 oyster farms distributed along the NSW coast. Spat (25 000) of each ploidy were supplied to participating farmers between August and November 1994 and monitored regularly for growth and mortality until December 1996. Mortality data were collected for all sites (Hand et al. 1998) but only the data from seven sites from four estuaries (Lake Pambula (sites 1, 2 and 3), Woolooware Bay (Georges River farms 1 and 2), Brisbane Waters and Tilligerry Creek (farm 2)) that were identified as having oysters with winter mortality during the course of the study, are presented here.

Oysters in these four estuaries suffer regularly from winter mortality being in the mid to southern half of the state. Winter mortality in the oysters was diagnosed from the appearance of yellow to brown lesions on the labial palps, gills, mantle, adductor muscle and/or gonad (Roughley 1926). All participating farmers grew the diploid and triploid oysters using the same standard commercial methods; stocking densities were standardised within estuaries (Hand et al. 1998). As this study was based on commercial farming methods, oysters were graded at all sites when large size ranges developed to allow "normal" growth of small oysters by removing competition from larger oysters.

Initially, oysters from Georges River, farm 2 were divided between two leases in the same bay. As there was little difference in growth and mortality of oysters between the two leases after 12 months these oysters were recombined. Oysters at Lake Pambula were divided among three farmers (sites 1 to 3) when they reached a large enough volume (after 6 months at site 3).

As is commercial practice (Nell et al. 1988), oysters were moved where possible during winter to avoid infestation by *M. roughleyi*. Oysters from both Georges River farms were moved to up-river leases from May/June through to November/December during the second winter (1996). Tilligerry Creek oysters were moved up-river in August and were returned in November during the first winter (1995) and August to December during the second winter (1996).

An additional comparison to the hatchery diploids and triploids was possible at the Brisbane Waters' site with wild-caught diploids (provided by the farmer) grown under the same conditions as hatchery stock.

Sampling Strategy

Being a commercial-scale study, the large number of oysters at each site (50 000 in total) made sampling of all stock logistically difficult. For this reason, mortality was assessed from a random 10% (by volume) of stock every two months until November 1995 when the sampling frequency was changed to once every three months. Where several grades were present a weighted mean of mortality was determined (see Statistical Analyses). Oyster shells from dead oysters were returned to trays/baskets after each sampling episode (unless all stock was brought in for sampling) to maintain equivalent apparent mortality between sampled stock and stock remaining on leases. Total weight for both diploid and triploid groups was measured every two months for the first six to nine months of the study to assess overall losses of stock as well as proportions in each grade. As handling became more difficult with the increase in volume of stock, farmers were given the choice of bringing in either a random 25% or all stock every six months for measurement of total weight. Weights of dead oysters and overcatch were weighed and accounted for when total weights were measured.

Temperature and salinity were determined weekly by farmers using a thermometer and hydrometer. Salinity values were calculated using a density/temperature conversion table (Wolf and Collins 1979).

Statistical Analyses

Monthly percent mortality is presented as a weighted mean when oysters were in several grades:

$$\overline{X_w} = \frac{\sum^{n} W_i \overline{X_i}}{\sum^{n} W_i}$$

where $\overline{X_w}$ is the weighted mean of mortality calculated from the mean, $\overline{X_i}$ of each of n grades, and each $\overline{X_i}$ weighted by a factor W_i (Sokal and Rohlf 1995). The total number in each grade was used as a weighting factor and was estimated from the total weight of all oysters for each grade divided by the mean weight of individual oysters for that grade (Hand et al. 1998). Cumulative percent mortality was calculated for the period June-December for both years to determine the effects of winter mortality, which are generally expressed from June to October (Nell and Smith 1988) (as sampling was 3 monthly, November and December were included). Monthly mortality (frequency dead/alive) and cumulative mortality were analysed for association with ploidy level using Chi-squared contingency tables (Sokal and Rohlf 1995).

Initial shell heights of hatchery diploids and triploids and wild-caught diploids were compared using analysis of variance (Sokal and Rohlf 1995) after homoscedasticity was confirmed with Cochran's test (Winer 1991).

RESULTS

For all seven sites, signs consistent with the disease winter mortality (see *Oyster Management*) were found in both diploid and triploid oysters. Salinity and temperature data are shown in Fig. 3.

Tilligerry Creek

Winter mortality signs were noted in diploids and triploids from Tilligerry Creek in August 1995 but mortality was low (<2%) for both ploidy types (Fig. 1) with no significant effect of ploidy on mortality ($p > 0.05$). However, there was a significant association between ploidy and cumulative mortality over the winter/spring of 1995 with higher mortality of diploids compared to triploids (Table 1). Oysters were moved in August of both years to "over-winter" at a site in Port Stephens (Tea Gardens) where they have previously been less prone to the disease (unfortunately salinity and temperature data are unavailable for the Tea Gardens site). Although some mortality occurred (Fig. 1), winter mortality signs were not evident during the winter/spring of 1996. Mean temperatures and salinities (where data are available) indicate generally cooler temperatures

(13.8ºC c.f. 15.5ºC) and similar salinities (27.3‰ c.f 27.2‰) for the winter of 1995 compared to 1996.

Brisbane Waters

When compared to hatchery diploids and triploids, wild-caught diploids were found to have significantly ($p < 0.05$) larger initial shell heights (21.5 mm compared to 9.8 and 9.7 mm for diploids and triploids respectively, n=400; data were heterogeneous). Wild-caught diploids suffered significantly higher mortality ($p < 0.001$), associated with winter mortality signs, than hatchery diploids and triploids during the winter of 1995 with a mortality rate of 51.5% for the period August-September compared to 3.1 and 0.7% respectively (Fig. 1). Insufficient numbers of wild-caught diploids remained after the first year to continue the comparison. Mortality of hatchery oysters, associated with winter mortality signs in oysters at the Brisbane Waters site, was high during both the 1995 and 1996 winter/spring seasons (Fig. 1). There was a highly significant effect of ploidy level ($p < 0.05$) on cumulative mortality between June-September, 1995 and 1996 (Table 1). Monthly and cumulative percent mortality rates of diploids during winter/spring were consistently higher than that of triploids. For example, cumulative mortality of diploids during the 1996 winter/spring period was 76.8% compared to only 16.9% for triploids. Mean salinity over winter at this site was lower (26.6‰) in 1995 than 1996 (29.2‰) whilst temperature was slightly higher for the 1996 winter season (14.2ºC c.f. 13.6ºC).

Georges River: Farm 1

No winter mortality signs were noted in either diploid or triploid oysters during 1995 with no significant effect of ploidy level on cumulative mortality. Winter mortality was noted in diploids and triploids in September 1996 (despite having been moved upriver in May) with a highly significant ($p < 0.001$) effect of ploidy on cumulative mortality (40.0 and 18.1% for diploids and triploids, respectively) (Table 1). Both mean temperature and salinity were slightly lower during the 1996 winter compared to the 1995 winter at this site at 13.9ºC and 31.0‰ c.f 13.6º and 29.5‰ for 1995 and 1996, respectively.

Georges River: Farm 2

Winter mortality signs were evident in both diploids and triploids during both 1995 and 1996 winter/spring seasons with generally higher mortality in diploids than triploids (Fig. 1). This was particularly evident during the second winter (1996) with cumulative mortality of 45.0% of diploids compared to only 14.2% of triploids (Table 1). Ploidy level had a significant ($p < 0.01$) effect on cumulative mortality over winter/spring for both years (Table 1). There was little difference between mean salinity over winter 1995 (29.2‰) and 1996 (29.5‰); similarly, mean temperature over winter in 1995 was 13.2ºC compared to 13.6ºC over the 1996 winter season.

Lake Pambula: Sites 1-3

Winter mortality signs were observed during the second winter corresponding to an increase in monthly mortality in both diploids and triploids at all three sites (Fig. 2). The effect on cumulative mortality was greatest at site 1 with 49.9% of diploids and 16.4% of triploids dying between June and December 1996. For all three sites there was a significant association ($p < 0.01$) between ploidy level and mortality with a higher cumulative mortality of diploids occurring in all cases (Table 1). A small increase in mortality of diploids was noted in March 1996 at site 3 but not at the other two sites. Mean winter temperatures at Lake Pambula were lower than the more northern sites for both 1995 and 1996 (11.9 and 12.8ºC respectively). Salinity was high compared to other sites and similar for both winter seasons (32.9 and 32.8‰).

DISCUSSION

The high mortality of wild-caught diploids at the Brisbane Waters site during the first winter season (1995) compared with relatively low hatchery diploid and triploid mortality, may be attributed to the greater mean size of wild-caught oysters. Larger oysters are known to be more

susceptible to winter mortality (Wolf 1967). The difference in susceptibility of diploids and triploids to winter mortality was most pronounced at this site, and greater in 1996 than in the 1995 winter. Despite the cumulative mortality of triploids (16.9%) being similar to that of triploids at the other sites (mean: 12.2%; range 4.0-18.1%), visual inspection of a sample of hatchery diploids at this site showed they were severely affected by the parasite (76.8% cumulative mortality). Oysters at Brisbane Waters were not moved over winter to avoid infestation (in contrast to oysters at both Georges River sites and Tilligerry Creek) accounting for the higher mortality of diploids. However, this does not explain the relatively low mortality of triploids at Brisbane Waters. A similar trend of greater triploid survival relative to diploids through outbreaks of winter mortality was found for all sites: average cumulative mortality over winter/spring, 1996 of diploids across all seven sites was 35.0% (range 6.7-76.8%) compared to only 12.2% for triploids (range 4.0-18.1%) despite farmers at three of the sites using standard disease management strategies (i.e. moving stock during winter/spring). Average mortality of triploids at the eight unaffected sites in the associated farming study was 6.1% during the same winter/spring period and was 7.0% for diploids. Triploids appear to have some inherent property of that enables them to cope better than diploids with infestation by *M. roughleyi*. However, triploids are not resistant to infestation by *M. roughleyi* and do not always survive outbreaks of the disease better than diploids. Nell et al. (1994) compared diploid and triploid Sydney rock oysters at a site in Woolooware Bay, NSW and found no significant difference in mortality between May and December (winter-spring). Similarly, although data are not available, high mortality with signs of *M. roughleyi* infestation, of both diploid and triploid Sydney rock oysters was observed at a site in Lake Merimbula, NSW over winter 1996 (P. Maguire pers. obs., oyster farmer, 1996).

Cold winter temperatures and high salinity are the main factors associated with increased mortality from *M. roughleyi* infestation (Wolf 1967) and this is generally supported by the conditions prior to and during outbreaks in the present study. Most mortality occurred when mean winter water temperatures were 14ºC or less and mean salinity 29‰ or higher. Physical conditions do not however, explain the particularly severe outbreak of winter mortality at Brisbane Waters in 1996. Conditions of temperature and salinity at the Brisbane Waters site in 1996 were not particularly unfavourable compared to the remaining sites yet 77% of hatchery diploids died (c.f. 17% of triploids).

Winter mortality generally affects the larger oysters in a population (Wolf 1967). This may simply be due to the greater volume of water filtered by larger oysters increasing the likelihood of being exposed to the parasite or a cumulative effect of parasite exposure in older/larger oysters. Many farmers also believe that oysters with high meat condition are more susceptible to the disease. The increased survival of triploids compared to diploids is therefore contradictory, as triploids in the associated farming study (Hand et al. 1998) were on average 30% heavier and generally had better meat condition over winter than sibling diploids (R. E. Hand unpublished data). A comparison of mortality and growth data suggests a size-threshold for both diploids and triploids above which they are more susceptible to the disease. That is, when exposed to *M. roughleyi*, mortality seemed to be greater in oysters at or above a mean size of 40 mm shell height and 10 g whole oyster weight. Although this is an approximate size only and smaller oysters are still affected (to a lesser extent) by the disease, it is similar to the size at which triploid Sydney rock oysters show a growth advantage over diploids (Hand et al. 1998); that is, when diploids are diverting a greater proportion of their energy to gametogenesis. This may indicate a relationship between the state of gonad development (or gonad composition) and the effect of winter mortality on oysters and is supported by the fact that diploid oysters of better meat condition are more susceptible to the disease. As triploids had a higher meat condition than diploids over winter it is likely that the changes in gonad composition of diploids with increasing size (as opposed to meat condition) is influencing susceptibility to the disease. That is, the different composition of the triploid gonad due to limited gamete development (and similar to the immature diploid) may either confer an advantage to the oyster when challenged by the disease or discourage initial infestation.

The protistan parasite *Perkinsus marinus* causes the disease ‘Dermo’ in American oysters, *Crassostrea virginica*. The proportions of phospholipids and specific fatty acids in *P. marinus*, compared to the host oyster, indicate possible active assimilation of fatty acids from the oyster

(Volety et al. 1995). *P. marinus* has also been shown to cause a reduction of up to 40% of free amino acid levels in oysters (Paynter 1996) which disturbs the animal's osmoregulatory capacity. Fatty acids, lipids and amino acids constitute important energy reserves to animals during periods of low food availability (Stryer 1988) such as are experienced in winter in NSW (Roughley 1926). *M. roughleyi* infested oysters are often characterised by an inability to maintain valve closure when out of water. This has usually been attributed to the appearance of lesions in the adductor muscle (Roughley 1926). The same symptom in *P. marinus* infested oysters has been attributed to lower glycogen levels in the adductor muscle of infested oysters (Dwyer and Burnett 1996). Glycogen levels in diploid oysters generally fluctuate throughout the year with a similar pattern to condition index. Levels over winter are generally low following spawning in summer/spring; in contrast, triploids maintain relatively stable glycogen levels throughout the year (Nell et al. 1994). Smith (1991) found no difference in glycogen levels in *S. commercialis* with varying degrees of winter mortality although a general decline occurred over winter and glycogen was measured on a whole meat basis rather than in individual tissues such as the adductor muscle. *M. roughleyi* may have a similar effect to *P. marinus* of reducing localised energy stores when whole body levels of glycogen are generally low. In addition, a disturbance of free fatty acid and amino acid levels during winter (when lipids and amino acids are important sources of energy) would have serious physiological consequences which triploids may be able to avoid having higher glycogen stores available. Infestation may also be related to initial free fatty acid levels which would be higher in overmature diploids. That is, oysters which retain developed gametes for an abnormally long period suffer changes in lipid metabolism leading to abnormally high levels of free fatty acids in the gonad (Mori 1979). Oysters are more likely to retain gametes through to winter during years of cold winter temperatures and low rainfall (conditions that are also known to increase the incidence of winter mortality) (Nell and Smith 1988).

In conclusion, although triploid Sydney rock oysters do not always survive *M. roughleyi* infestation better than diploids, in the present study triploidy had a significant ($p < 0.01$) effect on mortality at six of the seven sites where winter mortality occurred during the final year on leases. If these results could be applied on a broad scale to estuaries where winter mortality accounts for a major proportion of stock losses and costs, farming of triploid Sydney rock oysters could substantially improve the profitability of commercial oyster farming in NSW. That is, farming triploid Sydney rock oysters using standard disease management practices would on average reduce the loss of stock to winter mortality by 65%, in the final year on leases (when oysters are most valuable as well as normally being more susceptible to the disease).

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Table 1. Cumulative mortality[a] of diploid and triploid Sydney rock oysters, *Saccostrea commercialis*, over two consecutive winter/spring seasons in estuaries where the disease winter mortality occurs.

Farm	June-December, 1995 - Cumulative mortality (%)				June-December, 1996 - Cumulative mortality (%)		
	diploid	triploid	wild-caught diploid[b]	ploidy effect[d]	diploid	triploid	Ploidy effect[d]
Tilligerry Creek (farm 2)[c]	5.3	1.9	-	*	6.7	7.2	n.s.
Georges River (farm 1)[c]	3.2	3.0	-	n.s.[e]	40.0	18.1	***
Georges River (farm 2)[c]	8.1	3.4	-	**	45.0	14.2	***
Lake Pambula (site 1)	4.4	3.3	-	n.s.[e]	49.9	16.4	***
Lake Pambula (site 2)	1.6	1.0	-	n.s.[e]	11.1	4.0	**
Lake Pambula (site 3)	2.5	1.6	-	n.s.[e]	15.2	8.3	**
Brisbane Waters	7.0	2.9	53.9	***	76.8	16.9	***

[a]Cumulative % mortality for the period June to December for both years.

[b]Spat provided by farmer had a larger initial size than hatchery stock; discontinued in first year due to insufficient numbers.

[c]Stock at Tilligerry Creek and both Georges River farms was moved upriver during winter.

[d]Chi^2 test of association of ploidy and number dead/alive: n.s. = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

[e]Frequency was less than 5 in >20% of 2x2 contingency table cells so results should be interpreted with caution.

4.5. Hand, R.E., Nell, J.A., 1999. Studies on triploid oysters in Australia. XII. Gonad discolouration and meat condition of diploid and triploid Sydney rock oysters (*Saccostrea commercialis*) in five estuaries in New South Wales, Australia. Aquaculture 171, 181-194.

Studies on triploid oysters in Australia. XII. Gonad discolouration and meat condition of diploid and triploid Sydney rock oysters (*Saccostrea commercialis*) in five estuaries in New South Wales, Australia

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Abstract

The relative performance, in terms of meat condition, of triploid compared to diploid Sydney rock oysters varied among five widely distributed sites in New South Wales. Over the final year on leases, ploidy, month and the ploidy*month interaction had a significant effect on meat condition at all sites except for ploidy at the southern, Lake Pambula site. From March to December (autumn to the first month of summer) triploid condition indices were higher, or not significantly different from those of diploids at all sites. Condition indices of triploids were higher than those of diploids from May to November/December at four of the five sites. A higher condition index of triploids became apparent later in the sampling period for the Lake Pambula site in comparison to the remaining four sites.

Triploid Sydney rock oysters were susceptible to brown discolouration of the gonad surface. Discolouration occurred in localised areas of the gonad and was not correlated to condition index except for triploids at Lake Pambula. As discolouration was less noticeable during cooler months of the year, thus coinciding with the generally superior condition of triploids relative to diploids during winter and spring, triploids remain a viable winter crop for farmers throughout New South Wales.

Keywords: Condition Index, Discolouration; Oysters; Triploid; Gonad Colour

1. Introduction

Triploid Sydney rock oysters (*Saccostrea commercialis*) in New South Wales (NSW) have been proposed as a commercial alternative to diploids due to their superior growth rates and prolonged marketability (Nell et al., 1994; Hand et al., in press, a). Specifically, triploids have potential as a winter crop in NSW where Sydney rock oysters are frequently unmarketable following the spawning season in summer/autumn (Nell, 1993; Nell et al., 1994).

Although the study of Nell et al. (1994) showed higher condition indices for triploid Sydney rock oysters over the last 10 months of culture on leases, this study was limited to a single estuary (Port Stephens). The magnitude of the difference in condition index between diploids and triploids varied among leases within Port Stephens. Similarly, condition indices of diploid and triploid Pacific oysters *Crassostrea gigas* at three sites in Tasmania varied greatly with consistently lower triploid than diploid condition indices at one site (Maguire et al., 1994b). Sydney rock oysters are farmed in 35 waterways in NSW (Nell, 1993); to be a commercial proposition, triploids need to show an advantage over diploids in terms of meat condition in estuaries other than Port Stephens.

Organoleptic comparisons of diploids with triploids have previously shown few differences in consumer acceptance between the two ploidy types. For both Pacific oysters and Sydney rock oysters, consumers either prefer triploids or perceive little difference between the two (Allen and Downing, 1991; Maguire et al., 1994a; Nell et al., 1994). However, it was noted during an associated farming study (Hand et al., in press, a) that triploid Sydney rock oysters were particularly prone to localised

discolouration of the gonad. This discolouration differed from the grey gonad patchiness frequently seen in diploids following partial spawning. Rather, triploid Sydney rock oysters developed distinct light to dark brown patches on the gonad surface. This has also been an occasional problem with triploid Pacific oysters farmed in Tasmania (Maguire et al., 1994a).

Gonad appearance and condition are important factors in marketability of oysters as the gonad is visually the most obvious organ of a shucked oyster and largely influences the overall flavour and texture of the meat. To compare diploid and triploid Sydney rock oysters in different areas of NSW, an index of meat condition was monitored over the final year on leases in five widely distributed estuaries. Concern from oyster farmers that the gonad colour of triploids would affect their marketability, prompted the monitoring of triploid and diploid gonad discolouration at these sites during the final nine months (spanning the final winter season).

2. Materials and methods

2.1. *Larvae and spat production*

Sibling diploid and triploid oysters were produced in February 1994 in Port Stephens, NSW as part of an extensive farming study. Triploidy induction, larval and spat rearing are described in more detail in the associated farming study paper by Hand et al. (in press, a). The ploidy of cytochalasin B treated oysters was determined by flow cytometry at 10 weeks post-settlement and found to be 88% triploid.

2.2. *Oyster management and site selection*

25 000 diploid and 25 000 triploid oysters were supplied to the five farms (Fig. 1) from July to November 1994 (Table 1) as they reached a suitable size for ongrowing (7-10 mm shell height). At each estuary, diploid and triploid oysters were grown under the same conditions using standard culture techniques for the particular farm involved. Culture methods used at each site are summarised in Table 1. Where possible, stocking densities were standardised among sites at 2 l of seed oysters for cylinders (Nell, 1993) and 50% coverage for tray culture. Oysters at Georges River and Tilligerry Creek sites were moved during winter to lower salinity areas within the same estuary to reduce infestation by the protistan parasite *Mikrocytos roughleyi*, which causes the disease "winter mortality" (Farley et al., 1988).

2.3. *Oyster sampling and analysis*

Sampling commenced during the second year on leases. 24 diploid and 24 triploid oysters (4 replicates of 6 oysters) were sampled from each lease every month from December 1995 to December 1996. Where several size grades existed (at all sites but Tilligerry Creek and Georges River), samples were taken from the middle grade only. Whole oyster weight was determined before each oyster was opened. Oysters were opened using a stainless steel knife, the meat was removed and any shell debris or other contaminating matter rinsed from the meat with distilled water. It was then blotted dry and wet weight determined. The shell was rinsed and dried then weighed. The meats were dried at 90ºC for 48 h then transferred to desiccators to cool before determining final dry weight. Condition index (CI) was calculated using the following formula (Crosby and Gale, 1990):

CI = dry meat weight (g) x 1000/cavity volume (g) where:
cavity volume = whole weight (g) - shell weight (g) (Lawrence and Scott, 1982).

From April 1996, the gonad of each oyster sampled was scored for localised gonad discolouration using the progressive scoring criteria in Table 2 that ranged from 1 for "normal" gonads to 5 for highly discoloured gonads.

2.4. *Statistical analysis*

Condition indices and gonad discolouration scores are shown in the figures as mean±95% confidence intervals (n=24). Condition index data (n=4 replicates) were analysed using a two factor (ploidy and month) ANOVA on GMAV5 software (Institute of Marine Ecology, University of Sydney, NSW). Homogeneity of variances was checked using Cochran's test and multiple comparisons made with the Student-Newman Keuls procedure (Winer et al., 1991). Correlation of condition indices and gonad discolouration scores was determined by Spearman's rank order correlation (Sokal and Rohlf, 1995).

3. Results

3.1. *Condition index*

Ploidy, month and the ploidy*month all had a highly significant effect (P<0.001) on condition indices (Fig. 2) of oysters at all sites (except ploidy at Lake Pambula; P>0.05). However, data for the Hastings River, Tilligerry Creek and Hunter River sites should be interpreted with caution as variances were heteroscedastic (P<0.05). Data are only available until November, 1996 for the Lake Pambula, Tilligerry Creek and Georges River sites as oysters were being marketed by farmers in December. At the commencement of sampling, during late spring/summer (November-January) of 1995/96 diploids at Georges River and Lake Pambula sites had higher mean condition indices (hereafter termed condition indices) than triploids (Fig. 2). At the Tilligerry Creek site, condition indices of diploids and triploids were similar over the summer of 1995 (Fig. 2) whilst at the Hunter River site condition indices were higher for triploids from December 1995 to November 1996. There was considerable difference between diploid and triploid condition indices at the subtidal Hastings River site with significantly greater mean triploid condition indices throughout the sampling period (P<0.05); however, diploids had generally low condition indices at this site relative to diploids from other estuaries (Fig. 2). Condition indices (Fig. 2) were higher (or not significantly different, P>0.05) for triploids compared to diploids for the final 10 months on all leases from March to December (autumn to spring/early summer). At four of the five sites, triploid condition indices were higher from May to November. Where comparative data are available, the relative condition of triploids compared to diploids generally appeared to improve during the spring/summer of 1996 compared to the previous year's spring/summer.

3.2. *Discolouration of gonads*

Gonad discolouration was particularly noticeable in triploids when compared to diploids at all sites excluding the Hastings River (Fig. 3). Oysters at Hastings River were affected by an unidentified disease which caused red discolouration in both soft tissue and interior valve surfaces and subsequent high mortality. These blemishes were more noticeable in diploid than triploid oysters and often associated with poor gonad condition and gonad discolouration (Fig. 4A).

Between August and November the difference in discolouration indices between diploids and triploids was generally less noticeable than during other months (Fig.'s 3 and 4B). From the data available and through visual monitoring, triploids appeared to develop more obvious discolouration during the summer and autumn months (Fig.'s 3 and 4C) particularly towards the end of the spawning season (unpublished data, 1997). Highest mean discolouration indices occurred in autumn (April or May) at all five sites (data for summer are not available). Although the gonad discolouration was different from that described for spent diploid oysters, it is possible that some diploids with poor or patchy gonad condition may have been incorrectly classified thereby accounting for the increase in diploid discolouration during the cooler months. A transverse section through the gonad of triploid oysters with level 3 to 5 patchiness revealed discolouration extending below the surface of the gonad to varying depths and to a distinct level; that is, there was no gradation from brown to "normal" coloured gonad. No samples were found with discolouration extending through the entire depth of the gonad.

Condition index was not correlated with gonad discolouration at any site (Table 4), except for Lake Pambula which showed a significant ($P<0.01$) inverse relationship for triploids but not for diploids.

4. Discussion

It has been evident for several years that performance of triploid shellfish in terms of their growth advantage over diploids is dependent on the environment (Shpigel et al., 1992; Allen, 1995). The advantages (or disadvantages) of triploid over diploid oysters in terms of meat condition have also been shown to vary according to growing conditions such as temperature (Shpigel et al., 1992), food availability (Davis, 1989) and site (Maguire et al., 1994b). This is reflected in the variation among the five sites monitored in the present study where the benefits of triploidy in terms of meat condition appeared to be less pronounced at the southern (cooler) sites. For example, ploidy, month and ploidy*month all had a significant effect on condition index at all sites except for ploidy at the southern, Lake Pambula site. Nonetheless, triploid condition indices were higher, or not significantly different from those of diploids, at all sites from March to December and higher than diploids from May to November/December at four of the five sites. Triploid culture was found to be particularly suited to the northern, subtidal site in the Hastings River. In contrast, the difference in condition indices at the southern Lake Pambula site did not become apparent until later in the year (July) probably due to the slower growth of oysters and seasonal lag in temperature at this site. Winter, and to a lesser degree spring, are seasons often associated with reduced marketability of oysters following spawning in summer/autumn. The generally superior condition of triploids during winter and spring further demonstrates the suitability of triploids as a winter crop for farmers throughout NSW.

To our knowledge, tissue discolouration of triploid oysters has only been reported once previously by Maguire et al. (1994a) who noted that 5.9% of triploid Pacific oysters grown in Tasmania (but not diploids) developed brown patches on the meats in summer. Similar to the effect seen in Sydney rock oysters, pigmentation was only associated with surface tissue. No reference was made to discolouration at any other time of the year or to the relative degree of discolouration among oysters and sites.

Discolouration indices of Sydney rock oysters appeared to increase during the warmer months of the year, particularly during the post-spawning season of diploids, and was generally less obvious during winter/spring. Because of the apparent seasonality of discolouration, it was initially thought it may be associated with condition index in triploids. On the contrary, our analyses showed no correlation between the degree of gonad discolouration and condition index for any site, except for the southern, Lake Pambula site where a negative correlation for triploids was detected. Limited literature is available on the effect of ploidy level on meat colour. Triploid rainbow trout have been shown to differ from diploids in the colour attribute “chroma” (saturation of the perceived colour) of muscle tissue (Choubert et al., 1997) as well as in luminosity of flesh (Choubert and Blanc, 1985).

Live oysters (*Crassostrea virginica*) form brown cells in response to contaminant exposure and stress (Zaroogian and Yevich, 1993). These cells are possibly involved in detoxification and range in colour from light to black-brown in oysters from polluted sites. However, brown cells are generally associated with connective tissue rather than the gonad and do not explain the discrepancy between ploidy levels. Therefore, it is unlikely that the brown cells described by Zaroogian and Yevich (1993) are responsible for development of gonad discolouration in triploid oysters.

Meat discolouration is a common problem with shucked and processed oysters. Dried (Choi et al., 1977) and frozen (Jeong et al., 1990) oysters develop brown discolouration due to oxidative rancidity of lipids. However, lipid levels are similar in diploid and triploid bivalves (Shpigel et al., 1992; Utting et al., 1996) and oxidative rancidity is unlikely to cause discolouration in live animals. High storage temperatures of canned oyster meat are also known to cause brown colouration due to sugar amino condensation or the action of tyrosinase on free tyrosine (Lee et al., 1976). Differences in protein metabolism of meiosis 1 triploids compared to diploid oysters have been detected in the European flat oyster, *Ostrea edulis* (Hawkins et al., 1994) and in the Pacific oyster (meiosis 2

triploids) at high temperatures (Shpigel et al., 1992). Further investigation is required to determine whether the higher protein levels (hence different free amino acid equilibria) in triploid oysters combined with high temperatures during summer could cause a browning effect on the gonad.

The cause of the typically localised or patchy pattern of discolouration is also poorly understood. Despite being functionally sterile, limited gonadogenesis of triploid Sydney rock oysters does occur during the spawning season (Cox et al., 1996) and is frequently in localised areas of the gonad (unpublished data, 1996). Unfortunately no data are available for discolouration during the summer months. However, the generally higher gonad discolouration towards the end of the Sydney rock oyster spawning season (autumn) suggests an association between discolouration and the localised gonad development of triploids. Also of interest is the high level of polyploid mosaicism noted in triploids from the same batch used in an associated study (Hand et al., 1999). This is not the first time cytochalasin B induction of triploidy has had such an effect. Oysters (Allen et al., 1996) as well as salmonids (Allen and Stanley, 1979; Teplitz et al., 1994) are known to develop polyploid mosaicism following triploidy induction. Teplitz et al. (1994) evaluated mosaicism in various tissues of immature salmon and found increased levels in the gonads of triploids. Testes of naturally occurring populations of polyploid mosaic turtles (*Platemys platycephala*) contain both diploid and triploid cells although only the diploid cells produce gametes (Bickham et al., 1985). The patchy development of the triploid oyster gonad may be related to regions of diploid gonadogenesis, although, gonad discolouration is more frequently observed than mosaicism in triploids. Histological comparisons have been made between diploid and triploid oysters (Gardner et al., 1994; Cox et al., 1996); however, the ploidy of gonadal cells in mosaic oysters has not been thoroughly examined and warrants further research.

In conclusion, taste test panels have indicated no significant difference between diploid and triploid Sydney rock oysters in terms of appearance in spring (October) and summer (January) (Korac et al., 1996). Maguire et al. (1994a) reported significantly lower appearance scores for triploid Pacific oysters grown in Tasmania in late spring but not in summer or autumn. However, brown discolouration of the gonad surface, particularly during warmer months, does seem to be a phenomenon associated with triploidy in Sydney rock oysters and may affect their marketability during summer/autumn. One of the main advantages of triploidy in Sydney rock oysters, however, is their marketability (due to high meat condition) during the cooler months. Diploids are often in poor condition during winter and are more prone to the disease winter mortality (Hand et al., in press, b). As discolouration was less obvious or not apparent during winter and spring, and condition indices generally higher in comparison to diploids, triploids remain a viable winter crop for oyster farmers throughout New South Wales.

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Table 1. Culture methods for diploid and triploid Sydney rock oysters *Saccostrea commercialis* at 5 sites in New South Wales.

Estuary	Month Supplied (1994)	Nursery Culture		Growout	
		Culture method	Stocking density	Culture method	Management
Hastings River	July	Subtidal trays	50% coverage	Subtidal trays	Oysters dried out 1-7 days every 4 weeks
Tilligerry Creek	"	Intertidal trays	50% coverage	Intertidal trays	Moved during winter
Georges River	September	Cylinders	2 l	Baskets Intertidal trays	Moved during winter
Lake Pambula	October	Cylinders	0.5-2 l	Intertidal trays	
Hunter River	November	Cylinders	2 l	Baskets Intertidal trays	

Table 2. Graded scoring criteria for localised gonad discolouration of diploid and triploid Sydney rock oysters.

Score	Criteria
1	Gonad "normal" and consistent colouration or indistinct pale yellow patches covering less than 50% of gonad surface.
2	Distinct, pale yellow to pale brown patches frequently covering less than 50% of gonad surface.
3	Distinct fawn coloured patches.
4	Distinct brown patches on gonad frequently covering 50% or more of the gonad surface.
5	Distinct dark brown patches frequently covering 50% or more of the gonad surface.

Table 3. Results of 2 factor (ploidy and month) ANOVA[1] for condition index of diploid and triploid Sydney rock oysters *Saccostrea commercialis* at 5 sites in New South Wales.

Estuary	Ploidy		Month		Ploidy x Month	
	F	*P*	*F*	*P*	*F*	*P*
Hastings River	1754.236	0.000	42.190	0.000	15.848	0.000
Tilligerry Creek	34.484	0.000	17.980	0.000	3.645	0.000
Georges River	15.178	0.000	5.411	0.000	5.414	0.000
Lake Pambula	0.9548	0.332	39.658	0.000	5.705	0.000
Hunter River	67.756	0.000	11.551	0.000	3.667	0.000

[1]*n*=4 replicates of 6 oysters

Table 4. Correlation[1, 2] of condition index and gonad discolouration index of diploid and triploid Sydney rock oysters, *Saccostrea commercialis* at five sites in New South Wales.

Site	diploid r	triploid r
Hastings River	-0.45	-0.55
Tilligerry Creek	-0.48	-0.12
Georges River	-0.12	0.35
Lake Pambula	-0.26	-0.85**
Hunter River	-0.08	-0.36

[1]Spearman's rank order correlation, r.
[2] $^{**}P<0.01$

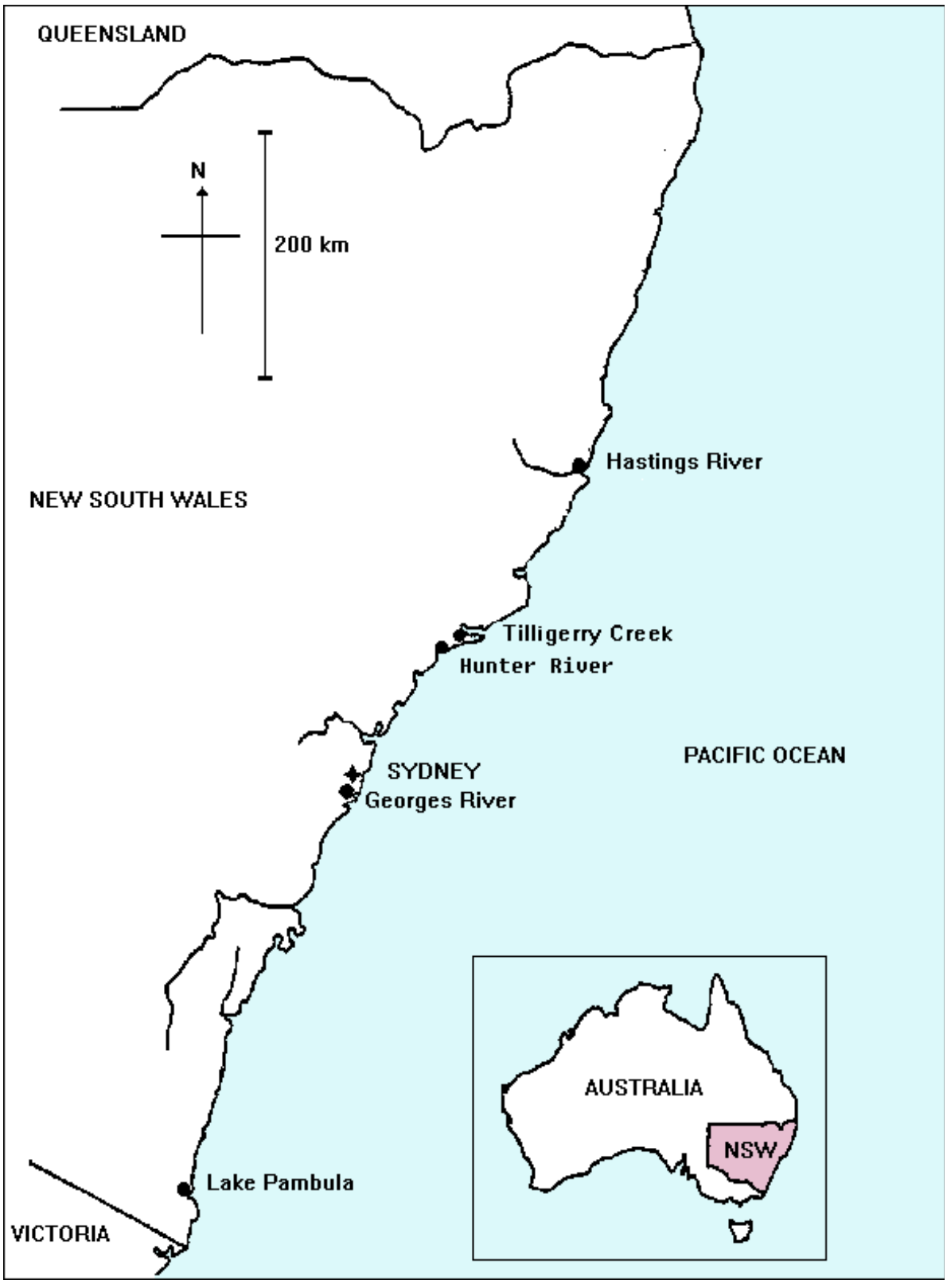

Figure 1. Estuaries in New South Wales where the five sites were located (adapted from Holliday et al., 1988).

Fig. 4. Photographs of diploid and triploid Sydney rock oysters, *Saccostrea commercialis* from three sites in New South Wales: the Hastings River in winter (A), Lake Pambula in winter (B) and Tilligerry Creek in summer (C).

4.6. Smith, I.R., Nell, J.A. The effect of growing height and growing method on winter mortality in diploid and triploid Sydney rock oysters *Saccostrea commercialis*.

The effect of growing height and growing method on winter mortality in diploid and triploid Sydney rock oysters *Sassostrea commercialis*

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Abstract

The response of Sydney rock oyster *Sassostrea commercialis*, triploids and diploids to winter mortality was compared under commercial growing conditions using both tray and stick culture in Lake Merimbula. The study demonstrated that tray oysters were not more susceptible to winter mortality than are stick-grown oysters. Thus farmers can use tray cultivation during winter at this location without having an increased risk of oyster kill. This result will need to be assessed in other estuaries along the central and southern NSW coast before the conclusions can be applied generally.

Keywords: Oysters; Mortality, Triploidy, Parasite; Culture

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1. Introduction

Triploid Sydney rock oysters, *Saccostrea commercialis* have shown growth performance advantages over their diploid siblings under commercial culture conditions (Nell et al, 1994, Hand et al., 1998a). In the mid to southern part of its commercial range the species frequently suffers high losses (Nell and Smith, 1988) from a disease known as “winter mortality”. This mortality is attributed to the protistan parasite *Mikrocytos roughleyi* (Farley et al., 1988). Whilst mortality can occur in winter, usually most of the oysters do not die until the warmer spring weather of September or October (Nell and Smith, 1988). The severity of kill can vary markedly between years, between estuaries, and even within leases.

Triploid production in hatcheries currently produces single unattached seed oysters which need mesh trays or similar methods for growout. For triploids to be commercially successful in NSW it is necessary that their resistance to winter mortality when grown on trays is at least as great as wild-caught oysters cultivated using the standard tarred stick method. Smith (1991) demonstrated no difference in response to winter mortality between Sydney rock oyster diploids and triploids on trays in the Georges River; however, the work of Hand et al. (1998b) shows triploidy confers greater survival when exposed to winter mortality compared with sibling diploids at some sites, but no difference at others.

Farmers can avoid this disease by increasing the growing height of oysters to 150 mm above the normal growing height, or moving their oysters to leases further upstream before the end of autumn (May), where lower salinities and higher growing heights offer some protection (Wolf, 1967, 1976; Smith, 1991). Dry autumns (high salinities) and early winters increase the likelihood of a severe kill (Wolf, 1967, 1976). Some farmers on the south coast of NSW, believe that single seed oysters are more susceptible to winter mortality than stick oysters. This disease is a regular and major killer of oysters on the south coast and if triploid technology is to be commercialised, farmers would have to be convinced that they do not take extra risks growing single seed triploid oysters. Thus an experiment was designed to compare the response of Sydney rock oyster triploids and diploids to winter mortality under commercial growing conditions for both tray and stick culture. A lower growing level was introduced as a third factor to increase exposure to winter mortality (Wolf, 1967, 1976; Smith, 1991). The trial was sited near the southern end of the

commercial range of Sydney rock oyster culture where winter mortality is most frequently encountered annually by oyster growers.

2. Materials and methods

2.1. *Experimental methods*

Juvenile sibling diploid and triploid Sydney rock oysters were obtained from the same batch used in the main commercialisation trials of this project (FRDC 93/151; Hand et al., 1998a). Parent stock were spawned at the Port Stephens Research Centre (PSRC), Mollusc Hatchery in February, 1994. Triploidy had been induced by blocking meiosis 2 polar body expulsion with cytochalasin B (Nell et al., 1996) which in this case resulted in 88% triploidy, as measured by flow cytometry on spat immediately prior to distribution to oyster farmers.

Spat were reared in mesh cylinders (Holliday et al., 1993), in Tilligerry Creek, Port Stephens to a size suitable for the experiment. Triploid and diploid oysters were dry graded with those which passed through 18 mm but retained on 14 mm mesh (diagonal measurement) used in the experiment.

An experiment to assess the effects of growing height, method and ploidy level was established at Merimbula Lake, NSW (36°55'S,149°55'E) in June 1994 and terminated in October 1996. It was located at the high salinity seaward end of the growing area to provide a greater risk of exposure to winter mortality disease and was serviced at approximately three month intervals to remove fouling organisms.

Treatments comprised two growing systems, two growing heights and two ploidy types providing eight treatment combinations each of which were replicated four times. Stick and tray replicates were each stocked with 400 oysters.

The "stick" growing system used standard tarred hardwood oyster sticks 25 mm square section x 2 m long in frames of 5 sticks nailed at 130 mm intervals to half stick cross members near each end (Nell, 1993). The sticks were used to support spat glued to the sides at 50 mm intervals using a non-acid curing silicone plumbers' sealant. This system models closely the traditional commercial growing stick method of wild oyster spat caught on tarred sticks (Nell, 1993) with the exception that oysters were glued at regular spacings rather than randomly settled from the wild.

The "tray" growing system used standard single seed trays of 1x2 m tarred hardwood timber frames, subdivided into 12 compartments, and the bottom covered with 9 mm (diagonal) polyethylene mesh. A second tray was used as a lid. Experimental tray replicates were stocked in two compartments initially, and only one side of the tray was used. The other side was stocked with a similar number spat of the same type for later histological sampling to test for the presence of *Mikrocytos roughleyi*.

Frames of sticks and trays were supported on a commercial oyster growing rack of tarred hardwood post and rail (Nell, 1993). Two growing height treatments were used. Standard growing height, at a little under half tide was the same as other commercial oyster leases in the area. The second growing height which used the same support materials was set up adjacent to the first but 300 mm below the standard height to increase exposure to winter mortality (Wolf, 1976).

Shell height (umbo to ventral valve margin) and whole weight were measured for 400 specimens randomly taken from each ploidy type before the sticks and trays were stocked at the start of the experiment. At termination in October, 1996 shell height and whole weight were measured for 50 oysters from each replicate. Mortality during the final winter was assessed by counting live and dead in each replicate at the termination of the experiment in October 1996. Percentage mortality was calculated as [no. dead/(no. dead + no. live)] x 100.

In June and October 1996, oyster samples were collected and forwarded to Dr R.D. Adlard at the University of Queensland, Department of Parasitology for histological examination (Adlard and Lester, in press) to determine levels of infestation with *Mikrocytos roughleyi*.

2.2 *Statistical methods*

All data were statistically analysed (Underwood, 1997) using multiple ANOVA in the statistical software package, Statgraphics, version 5 (Statistical Graphics Corporation, Rockville, MD). For the measurements on samples of spat taken at the start of the experiment, homogeneity of variances was confirmed for shell height for triploids and diploids using Cochran's test ($C=0.507$, $P>0.05$). Weights were log transformed to satisfy requirements for homogeneity ($C=0.501$, $P>0.05$).

Weight data were expressed as weight increase, (ie final mean whole weight for each replicate - initial mean weight,). Percentage mortality data were arcsin $x^{0.5}$ transformed. Homogeneity was confirmed for shell height and whole weight ($C=0.354$, $C=0.256$; $P>0.05$; respectively). Mortality remained slightly heterogeneous following transformation ($C=0.467$, $P=0.029$). This is, however, a minor departure from homogeneity and ANOVA is sufficiently robust to overcome such departures (Underwood, 1981).

3. Results

During the April 1995 visit, losses of oysters from diploid and triploid stick treatments were discovered on the high growing level. These apparently were caused, according to the leaseholder, by pelicans sometimes using the structure of the higher level treatments as a roost at low tides and led to markedly reduced numbers at termination for these treatments. It is probable that fouling organisms, especially on low trays reduced growth rates.

Winter mortality occurred only during the second growing season (June - October, 1996). Infestation of oysters with the winter mortality parasite, *Mikrocytos roughleyi* was evident in June and October 1996. (Table 1).

Shell height did not differ with ploidy when analysed using single factor ANOVA ($F=0.155$, $P>0.05$), however, in a multifactor ANOVA, shell height was significantly affected ($P<0.05$) by ploidy, substrate and by growing height, with a significant interaction ($P<0.05$) between substrate and growing height (Table 2). Whole weight increase was significantly affected ($P<0.05$) by ploidy and substrate, again with a significant interaction ($P<0.05$) between substrate and growing height. Triploids had significantly greater whole weight increase than diploids ($F=17.580$, $P<0.001$). Mortality was affected significantly ($P<0.05$) only by growing height.

4. Discussion

Winter mortality occurred only during the second growing season (June - October 1996) downstream, near the entrance in Merimbula Lake during this study. There was no significant affect of growing method on mortality, but oysters grown lower suffered greater rates of mortality. Diploid and triploid oysters in a parallel study carried out simultaneously further upstream experienced no winter mortality kill (Hand 1998a). There was, however, a similar growth advantage for triploids over diploids in this study as was observed in the parallel study by Hand et al. (1998a).

The study demonstrated that tray oysters are not more susceptible to winter mortality than stick oysters when grown at the same height. Thus farmers can use tray cultivation in winter mortality susceptible areas in Lake Merimbula without the worry of an increased risk of oyster kill due to

winter mortality. This effect may need to be assessed in further estuaries along the NSW south coast, before one can be sure that the results obtained in this study generally apply.

Acknowledgments

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Table 1. The proportion of Sydney rock oysters *Saccostrea commercialis* infested with the winter mortality parasite *Mikrocytos roughleyi* at Merimbula Lake.

Date	Treatment	No. examined	No infested	Proportion infested (%)
June 1996	Triploid/Tray/Low	30	0	0
"	Diploid/Tray/Low	30	0	0
Oct 1996	Triploid/Tray/High	20	6	30
"	Triploid/Tray/Low	20	0	0
"	Diploid/Tray/High	10	0	0
"	Diploid/Tray/Low	10	3	30
"	Triploid/Stick/High	10	3	30
"	Diploid/Stick/High	10	0	0
"	Diploid/Stick/Low	10	3	30

Table 2. The effect of growing height, method and ploidy level on shell height, weight increase and mortality of Sydney rock oysters *Saccostrea commercialis* in Merimbula Lake from June 1994 - October 1996.

Growing height	Growing method	Ploidy level	Shell height (mm)	Weight increase (g)	Mortality (%)
Low	Stick	Triploid	59.7±0.7	26.8±0.4	15.6±4.7
"	"	Diploid	57.0±0.2	23.0±0.4	18.4±6.0
"	Tray	Triploid	55.6±1.2	22.0±0.4	8.5±1.6
"	"	Diploid	53.5±0.8	18.6±0.6	16.5±3.8
High	Stick	Triploid	21.6±1.0	21.6±1.0	1.8±0.6
"	"	Diploid	54.1±0.7	20.4±0.5	2.5±0.5
"	Tray	Triploid	56.0±0.8	23.0±1.1	3.3±0.5
"	"	Diploid	52.7±1.5	19.3±1.0	8.6±1.2

4.7. Catt, C. An estimate of the economic benefits of farming single-seed triploid Sydney rock oysters compared to traditional single-seed diploid oysters.

An estimate of the economic benefits of farming single-seed triploid Sydney rock oysters compared to traditional single-seed diploid oysters

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Summary
Triploid oysters grow faster than traditional diploid oysters, whose three to four year production cycle involves considerable costs of establishment and production. By reducing the production time, triploid oysters offer a means to reduce costs. The reduction in costs is estimated to be about $2 per bag for each month by which production is reduced, but at least 6 months reduction would be required to offset the initial costs involved in switching from traditional natural stock to hatchery produced triploids. Additional benefits from selective breeding would include increased growth rates and disease resistance, as well as constant supply of spat throughout the year and consequent elimination of any off-season lulls in supply.

Aim
This report estimates the net financial advantage of faster oyster growth rates, which might be derived from the use of hatchery produced triploid oyster spat, compared to the traditional use of caught natural spat.

Background
Traditional oyster production is costly and time consuming. Establishment costs for medium sized oyster farms are in the order of $600,000, and because of the strong seasonality of natural oyster reproduction and the wide range of growth rates the production cycle is four years. Because the industry has been dependent on natural processes to provide the initial catch of young oysters (spat), research and development in the industry has traditionally concentrated on reducing establishment and running costs.

Over recent decades a number of different techniques have been developed to reduce the material and labour costs of oyster cultivation. However, because local circumstances favour particular methods of cultivation, because there has not been marked advantages between different methods, and because of the high costs of changing from one method to another, there is considerable variety in the methods and techniques of production.

Whilst most research and development has been aimed at methods of growing natural spat, considerable effort has also been applied to methods of breeding oysters in captivity, which offers several potential opportunities, such as increased growth rate, reduction in seasonality of production, and greater disease resistance and uniformity of production. One result has been the development of the means to produce triploid oysters. Growing more rapidly than the natural diploid oysters, they can cut the oyster production cycle from about three and a half years to less than three years.

Scope and basis of assumptions
Oyster production in New South Wales is carried out by businesses, which vary over a wide range of locations, sizes and methods of operations. Some growers catch all their own spat. Others buy and grow-on caught spat or hatchery-bred spat. Spat may be bought at different stages of growth, or may be bought in the traditional manner, on sticks, which buyers may grow-on, or may scrape or knock off the sticks and raise as individual 'single-seeded' oysters in a variety of containers.

In the absence of information on the current structure and practices of the industry, this report is based on an econometric model described in a draft report of 1991 (Catt, 1991; 1992), with allowance for changes in costs and prices, and increased efficiency of production up to December 1998. The report was based on a detailed study of traditional stick production and single seed / tray production

in Port Stephens which was carried out in 1991. From interviews with oyster growers, a detailed list of all operations was drawn up, and the amount and cost of materials, machinery and labour for each operation was estimated. Although the data in the 1991 study appeared to be the most comprehensive and up to date available, because the project was aborted before the model was fully verified, and as considerable change has taken place since the original study, the results of this approach may not reflect the relative costs and conditions of the current industry. This suggests that a detailed study of the current situation is warranted.

As single seed tray methods currently account for the majority of Sydney rock oyster production in New South Wales the report is based on an example of a medium sized oyster farm producing a yearly average of 200 bags (around 250,000 oysters) on trays from its own caught spat. Average production time using current methods is assumed to be 3.5 years. However, due to the seasonality of production, and the existence of a tail end of slower growing oysters, operations are carried out over a four-year cycle. Thus at any time throughout the year growers using these 'traditional' methods have four different cohorts of oysters on hand: the young spat spawned in the current year, and older groupings from each of the three previous years' spawnings. (See Fig. 1) Consequently, it takes some four years for all the returns on the initial money and effort invested in any batch of oysters to be achieved.

Previous attempts to increase the profitability of oyster production have focussed on reducing the costs of cultural operations. But as well as the costs imposed by the complexity of cultural operations, the lengthy growing period imposes considerable overhead costs on oyster production. Any reduction in the length of the growth cycle would therefore reduce the overhead costs of oyster production.

However, because of the seasonality of production, if the same average annual production is maintained, an effective reduction in overhead costs will not be gained unless the growing cycle is reduced sufficiently to allow the removal of a complete cohort of juvenile oysters from the growing stocks on hand. Effectively, if late summer/autumn-caught wild spat continue to be the source of new stock, this implies reducing the growing period sufficiently to reduce the growing stock on hand at any one time by a whole annual cohort. This appears to have already been achieved by some growers who have reduced their cycle of operations to three years by purchasing spat in spring and foregoing six to nine months of catching operations.

The use of cultivated spat offers the prospect of overcoming the strong seasonal factor inherent in the use of wild caught stock, whilst the faster growing triploid spat appears to offer the possibility of reducing the growth cycle to two and a half years, with a prospect of a further reduction to a two year growth cycle.

Impact of faster growth rates

Faster growth rates predominantly affect the overhead costs of oyster production, but have little effect on the variable costs, as the cultivation of faster growing varieties involves almost the same series of operations, and hence the same level of costs, but over a shorter time.

The overhead costs of oyster production consist of two main components: (i) the business service costs, which provide the framework within which the cultural operations are carried out, and (ii) the costs of depreciation in value - through wear and redundancy - of the improvements, equipment and machinery used by the business, which reflect the capital costs of establishment.

However, although faster growth rates would imply that the relatively constant overhead costs can be spread over a greater amount of production, resulting in a reduction in the cost of production per oyster, the benefits from this source are not likely to be very large. This is because the basic business service costs, such as telephone and other office expenses, tend to be a relatively small proportion of total overheads, whilst depreciation is more dependent on the use of facilities than on the time over

which they are used. So if the same use is being made of facilities in a shorter time, depreciation is unlikely to be significantly reduced.

From lack of information of the nature and range of current industry establishment costs, this analysis has considered only the steady-state situation of an established enterprise. Faster growth rates would benefit the establishment of a new business by reducing the time taken for the initial investment to achieve an initial return, and hence reduce the overall cost of any borrowings needed, or the opportunity costs of grower's capital invested in a new enterprise.

Benefits and costs expected from a change to triploid oyster production
By specifying the expected results of a change in production methods compared to existing practices, we can estimate the net benefit of the proposed changes. It is convenient to consider the expected results under four headings: (i) increases in income and (ii) decreases in costs, versus (iii) decreases in income and (iv) increases in costs. A comparison of the sum of (i) and (ii) against the sum of (iii) and (iv) for any existing and proposed situation then allows us to judge the potential financial benefit of the proposal.

In actual practice the benefits are unlikely to be achieved solely as increased production at the same cost, or as decreased costs for the same level of production, but rather through a mix of both. However, for simplicity, this analysis concentrates only on the likely decrease in costs at the same level of production.

1. Increases in income
Losses from mortality were assumed to be relative to the time involved. So shorter production times are likely to involve fewer deaths and result in increased numbers of oysters for sale. Consequently, on the basis of reduced time to harvest alone, the use of triploids is likely to yield an additional eight per cent of saleable oysters compared with the traditional use of diploids.

The potential for reduced losses from winter mortality in triploid oysters would lead to an increase in production from an initial number of spat. However, as limited information was available on the likely extent of reduction, no allowance has been made for this increase although, based on experimental work, a potential 65 per cent reduction in mortality has been claimed.
The potential for greater uniformity in size and shape from hatchery spat, as well as the elimination of any off-season in production, may increase the proportion of plate grade oysters, resulting in marginally higher average price. Again, no allowance has been made for this increase, due to lack of information.

2. Decreases in costs
Because roughly the same amount of work would need to be done, albeit in less time, reduction in the time required for oysters to grow to saleable size would not reduce the amount of labour needed nor the amount of wear and tear on machinery and equipment. Consequently, decreases in costs will not be in proportion to the reduction in the growing period. Instead, the difference in costs between different situations is greatly dependant on the way in which the growing stock are handled, and whether the requirements of individual annual cohorts overlap in the same season (requiring a greater investment in trays of the same mesh size) or whether they are handled in a way that minimises the total number of trays required.

As with any econometric model, depending on the periods of time which are assumed for the use of trays at each stage, and whether or not those periods overlap, small differences in management can result in major differences in the number of trays needed, and hence in the total cost of operations. These differences cause discontinuities, which give irregular changes in total cost between different lengths of turnover period. Figures 1 to 4 indicate the assumptions concerning tray management for the four situations used in the study, but many other permutations are possible.

A more rapid turnover will reduce the number of annual batches on hand and so reduce the size of lease required and the associated numbers of racks and trays. Compared with the $127,812 that leases, racks and trays would cost in the traditional four-yearly turnover, foregoing the catching operation by purchasing commercial spat to achieve a three-yearly turnover would reduce these costs to $125,477. The use of triploid spat to permit a 2.5 yearly turnover would further reduce these costs to some $86,353, and a two-year turnover would require only $77,390.

Assuming these expenses were made with borrowed capital at the commercial rate of 7.5 per cent, the savings in annual interest charges for each of these three alternatives would be $175, $3,110 and $3,782 respectively.

In addition to savings in interest on the capital costs of leases, racks and trays, the reduction in need for these resources will give a slight reduction in costs of repairs, maintenance, depreciation, rates and insurance, of, respectively, $134, $4,917, and $6,061.

The use of hatchery spat, whether triploid or natural, will reduce the cost of overheads by eliminating the need for current sources of spat. As no catching of natural spat will be required, an estimated saving of $1,114 will be made in annual variable costs of putting down and harvesting from sticks on catching leases, and a further $123 in annual maintenance, depreciation, insurance and rates on the appropriate area of catching lease and racks.

The potential for greater uniformity may decrease the amount of handling that must take place in grading oysters for market. However, no allowance has been made for this increase, due to lack of information.
Nor has any allowance been made for the decrease in risk, which a shorter production period should bring.

3. Decreases in income
There is some apparent potential for a reduction in triploid sales and prices due to the appearance of dark spots in the flesh of triploid oysters for significant periods during summer. As quantification of the market impact of this aspect requires considerable additional research beyond the scope of this study, no allowance has been made for this decrease due to non-marketability, and prospective suppliers may therefore prefer not to specialise solely in triploid production.

4. Increases in costs
The initial cost of purchasing triploid spat is the major cost of the proposed change in method of production. At a cost of 3 cents each, the 305,005 spat required each year to provide an annual output of 200 bags over 2.5 years, after an overall mortality of 0.5771 per cent per month (equivalent to 20 per cent loss over the traditional period of tray production) would be $9,150, whilst the 291,658 spat required for a 2 year cycle with the same mortality rate would be $8,750.

This compares with a cost of $3,148 to buy the 314,805 commercial diploid spat at 1 cent each needed to produce 200 bags over a 3-year period.

Discussion
On these assumptions, a change from using their own caught spat to using other sources of spat would save growers of a 200 bag a year operation about $1,546 in current annual running costs, but incur an additional cost of c.$3,148 if commercial spat is used. This marginal loss would, however, be offset by a reduction in the period of effective turnover to three years, as well as a reduction in risk and complexity of operation, which should enable greater production to be achieved from existing resources, as well as providing a number of benefits stated above which have not been measured.

The extra cost of triploid spat is likely to be entirely offset by the savings stated above to break even, whilst reducing the production period to about two and a half years. Further reduction in the

production period would bring further direct benefits of about $2 per bag for each month by which the production period is reduced.

Acknowledgment
The author wishes to express his thanks to the many people who took time to provide help, information and advice during the development of this study. The detailed practical comments of several oyster farmers, in particular, Geoff Diemar and Andrew Phillips, as well as the staff of the NSW Department of Fisheries, are gratefully acknowledged.

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NSW Fisheries (1999) Oyster Production Data from Oyster Farms in New South Wales: 1997/98

Attachment 1

Assumptions

No economic budgeting can be done without assumptions. The following assumptions were made in consultation with both growers and researchers.

1. The purchase price of hatchery spat was taken to be 3 cents per spat

2. The cost of purchased wild caught spat was taken to be 1 cent per spat

3. The average market price for:
   Plate grade oysters was taken to be $440 per bag at 100 dozen per bag
   Bistro grade oysters was taken to be $340 per bag at 110 dozen per bag
   Bottle grade oysters was taken to be $285 per bag at 130 dozen per bag

4. The problem of discolouration that is seen in triploid oysters, which may affect marketability if severe, was not taken into account.

5. The advantage of reduced kill from winter mortality in triploid oysters was not taken into account.

6. Losses between initial nursery deployment and sale were assumed at 20 per cent for the 'traditional' setup, and at an equivalent rate per month (0.5771 per cent) for the relevant period.

Definitions

The costs of running a commercial business are commonly divided into two kinds, "overhead" costs and "variable" costs.

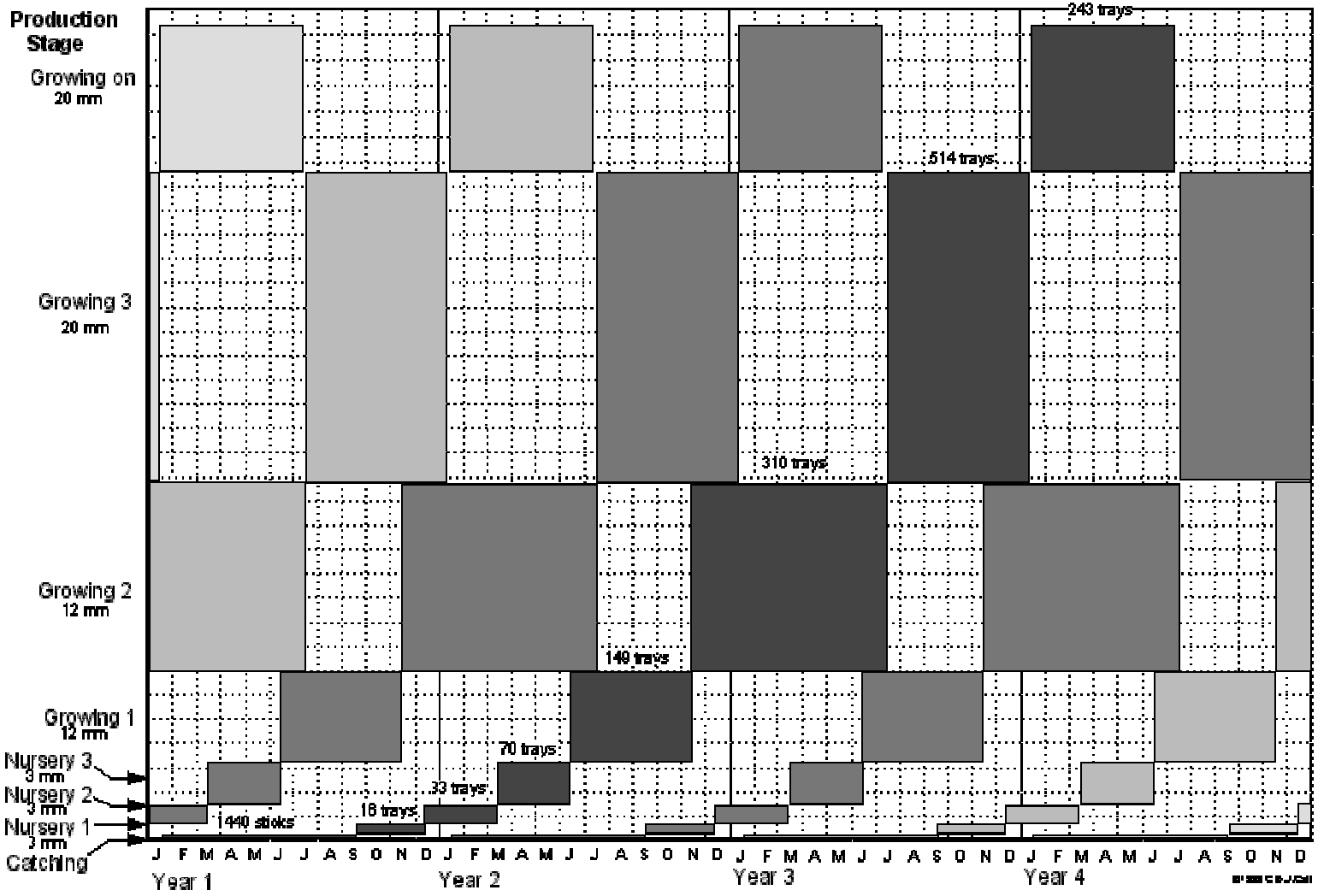
Overhead costs are expenses, which tend to remain constant throughout wide variations in the levels of a firm's activities over the short to medium term. They include **business service costs** such as telephone, postage, stationary, accounting, bank charges, rates, land tax, rents, leasing costs, and registrations. They also include **depreciation**.

Variable costs are expenses, which tend to vary in proportion to the level of activity of the business. They include labour, machinery operating costs, materials used for production and packaging, and freight costs. Repairs and maintenance are also primarily variable costs.

Depreciation is an accounting procedure to make allowance for the gradual loss, over several years, in the value of major items used in the business - such as structures, machinery, and equipment - through wear and tear and technical obsolescence. It is a capital cost, not an actual cash cost, but represents the drop in the walk-in-walk-out value of the business. It may be viewed as the amount that should be set aside to allow for the purchase of new capital items when the old ones finally wear out.

The **Opportunity Cost** of an investment is the net benefit that has been lost by rejecting the most profitable alternative. By choosing to invest in a particular business, people forego the opportunity to put their money into alternative investments.

Figure 1: Overlapping stages of successive 4-yearly batches of SRO from own caught spat



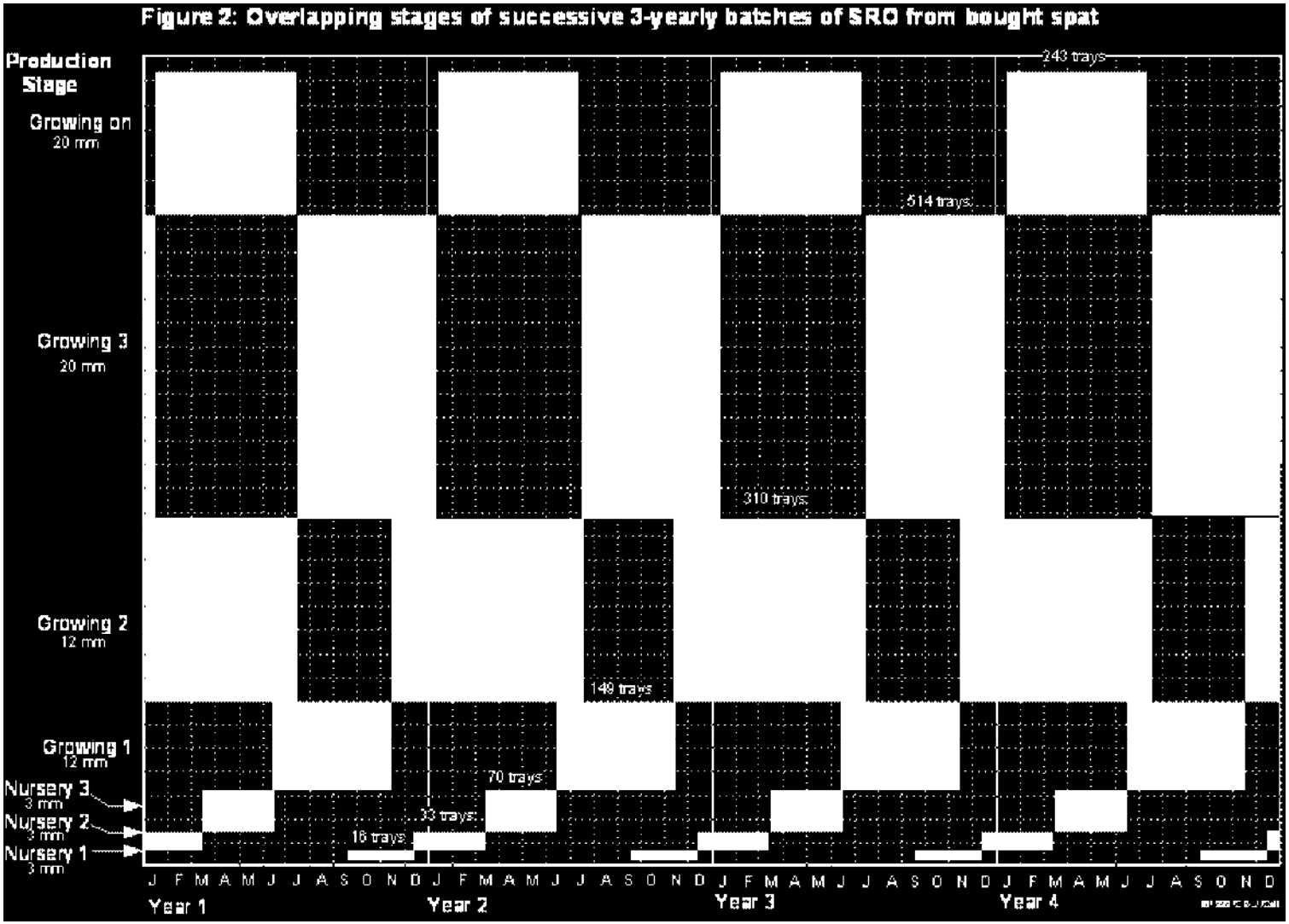


Figure 2: Overlapping stages of successive 3-yearly batches of SRO from bought spat
Production Stage
Growing on 20 mm
Growing 3 20 mm
Growing 2 12 mm
Growing 1 12 mm
Nursery 3 3 mm
Nursery 2 3 mm
Nursery 1 3 mm
243 trays
514 trays
310 trays
149 trays
70 trays
33 trays
16 trays
J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D J F M A M J J A S O N D
Year 1
Year 2
Year 3
Year 4

5. BENEFITS

NSW Fisheries established a commercial spat production program at its hatchery at the Port Stephens Research Center in November 1997. Around one million triploid Sydney rock oyster spat have been sold annually since then. It is expected that demand for triploid spat will increase with availability. Oyster farmers need to gain confidence that they will be able to rely on a regular supply of spat.

6. FURTHER DEVELOPMENT

NSW Fisheries established a Sydney rock oyster selective breeding program in 1990. Four selection lines were established for faster growth in Port Stephens. An average weight increase of 18% (3 months earlier to market) per line for third generation (two generations of selection) selection lines in Port Stephens was recorded in May 1997. The Sydney rock oyster breeding program was reorganised in 1996 and has received funding support from FRDC 'Selective breeding for disease resistance and fast growth in Sydney rock oysters'(Project Number 96/357) for the period January 1997 - December 2000. An experiment is planned for 1999/2000 to determine if it is possible to combine the faster growth of triploidy with that achieved with selective breeding (6 + 3 = 9 months earlier to market). If this is successful, the broodstock from the most advanced generation of the best breeding lines should be used for commercial triploid Sydney rock oyster spat production.

7. CONCLUSION

All objectives in section 4 were met. However, a proposed trial for use of caffeine to induce triploidy was replaced with the far more promising 6-dimethylaminopurine (6-DMAP). Triploidy can be readily induced using cytochalasin B (CB). Triploids grow faster than diploids in all situations once oysters have achieved sexual maturity. On average, triploid Sydney rock oysters reach market size (40-60 g) 3 months earlier (out of an average of 3.5 years) than their diploid siblings. Even the last triploid spat to leave the nursery are likely to out grow the fastest growing diploids. Triploids also hold meat condition longer over autumn and winter and suffer less winter mortality than diploids. Unfortunately triploids sometimes suffer from brown discolouration of the gonad surface. The incidence of this problem is unpredictable, but fortunately it has not been a major marketing problem and industry demand for triploid rock oysters is increasing.

8. APPENDICES

Appendix 1: Intellectual Property

The triploid program has not produced any confidential information and all data has been made freely available to the industry.

Appendix 2: Staff

New South Wales (Sydney rock oysters)

Name	Position	Institution	Time (%)	Qualifications
Dr John Nell	Prin Res Scientist	NSWF	20	Dip Appl Sc, Ph D
Mr Ian Smith	Scientific Officer	NSWF	20	B Sc
Ms Roz Hand	Fisheries Technician	NSWF	100	B Sc, Hons

Appendix 3: Related publications from this study

Triploidy publication series

1. Nell, J.A., Cox, E., Smith, I.R., Maguire, G.B., 1994. Studies on triploid oysters in Australia. I. The farming potential of triploid Sydney Rock oysters *Saccostrea commercialis* (Iredale and Roughley). Aquaculture 126, 243-255.
2. Nell, J.A., Hand, R.E., Goard, L.J., McAdam, S.P., Maguire, G.B. 1996. Studies on triploid oysters in Australia: Evaluation of cytochalasin B and 6-dimethylaminopurine for triploidy induction in Sydney rock oysters *Saccostrea commercialis* (Iredale and Roughley). Aquaculture Research 27, 689-698.
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4. Gardner, C., Maguire, G.B., Kent, G.N., 1996. Studies on triploid oysters in Australia. VII. Assessment of two methods for determining triploidy: Adductor muscle diameter, and nuclear size. Journal of Shellfish Research 15, 609-615.
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6. Hand, R.E., Nell, J.A., Reid, D.D., Smith, I.R., Maguire, G.B., 1999. Studies on triploid oysters in Australia: effect of initial size on growth of diploid and triploid Sydney rock oysters *Saccostrea commercialis* (Iredale & Roughley). Aquaculture Research 30, 35-42.
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