Development and delivery of technology for production, enhancement and aquaculture of blacklip abalone (*Haliotis rubra*) in New South Wales

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NON-TECHNICAL SUMMARY

2001/033	Development and delivery of technology for production, enhancement and aquaculture of blacklip abalone in NSW.

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OBJECTIVES:

- Produce and assess the utility of sterile triploid blacklip and/or hybrid abalone for fisheries enhancement, ranching and farming.
- Evaluate alternative methods of reducing high post release mortality rates commonly sustained by hatchery produced abalone seed.
- Develop indigenous community capacity to undertake the production of seed abalone and use of that seed for cost-effective and sustainable fisheries enhancement, ranching and farming of back-lip abalone.
- Produce economic models for enhancement of blacklip abalone fisheries using hatchery produced seed.

NON-TECHNICAL SUMMARY:

Introduction and background

This project is a revision of FRDC Project 2001/033 which was originally titled *Enhancement of the NSW blacklip abalone fishery using hatchery produced seed*. The original project included a significant cash contribution from the NSW Abalone Fishing Industry but unfortunately after FRDC approved the project, the industry decided against providing the cash contribution.

In consideration of this and of the wider potential benefits of the project, an alternative partnership was secured through the NSW Government's Indigenous Fisheries Strategy. The NSW DPI abalone aquaculture and enhancement project was originally initiated in the early 1990s by NSW south coast indigenous groups to develop abalone aquaculture for their communities so the indigenous community, through the Indigenous Fishing Strategy, were logical partners and key stakeholders in the R&D.

A precursor project (FRDC 98/219) had made good progress towards developing cost-effective fisheries enhancement and ranching technology for blacklip abalone in NSW. More than 20 million larvae were seeded over 12 locations and almost 900,000 "button size" juveniles at 50 locations. Preliminary economic modelling, incorporating seed production and deployment costs and size specific natural survivorship, identified "button size" (7 to 12 mm) 6 to 8 month old juveniles as those likely to be cost-effective for seeding depleted reefs in NSW.

Average survivorship from these releases was however much lower than that reported for equivalent size/age wild juveniles. The most probable cause was identified as high-density related

predation following release, possibly exacerbated by distinctive blue-green shell colouration and predator naivety of hatchery produced seed.

As stated above, indigenous groups had been actively seeking to establish hatchery based abalone farming, fisheries enhancement and ranching enterprises in southern NSW since at least 1993. The revised objectives and outcomes of this project supported this goal.

OUTCOMES ACHIEVED TO DATE

Objective 1 – Production and assessment of triploid abalone

Effects of two different chemicals cytochalasin B (CB) and 6-DMAP widely used to produce triploid molluscs (those with body cells possessing three rather than the usual two sets of chromosomes) were systematically assessed for the production of triploid backlip abalone. Optimal conditions for producing triploid black lip abalone including dose rate, application time and duration were identified for both chemicals. Large-scale hatchery production of triploid and diploid *H. rubra* was achieved and effects of triploidy on the various aspects of growth and breeding explored in a series of experiments.

Gonadal development and gamete maturation were abnormal in triploids that were effectively sterile. Female *H. rubra* triploids lacked visible gonads. By contrast male triploids formed similar sized gonads to diploid counterparts, but these suffered a brown-yellow discolouration and incomplete development of sperm. Growth and gonadal maturation of normal diploids occurred concurrently with the onset of sexual maturation in *H. rubra*. The shells of triploid *H. rubra* were shown to be longer and narrower than those of their normal diploid counterparts but the sex ratio of neither deviated from 1:1.

Improved flow cytometric techniques were developed for the verification of triploidy at various life phases of *H. rubra*. Up to three years of age no significant differences could be demonstrated in shell length, body weight, condition index and survivorship of sibling triploid and diploid *H. rubra*, except for a significantly larger foot muscle in triploid abalone. However over the ensuing 6 months major differences in overall shell size and total liveweight emerged in one particular group of triploids but not in other triploid groups nor in diploid controls. Further detailed discussion of these very encouraging results are constrained by non-disclosure provisions of a provisional patent registered by the FRDC and NSW DPI on behalf of stakeholders in April 2007. It can however be revealed that yields of saleable foot flesh of the particular triploid group referred to above was about twice that of all other groups at 3.6 years of age.

Objective 2 – Evaluation of factors affecting post-release survival of hatchery reared juveniles

Complimentary experimental programs were used to investigate seeding density and scale effects on post-release survival of hatchery reared juveniles.

Program 1

a) The first program assessed the impacts of several factors on post-release survival of cultured juvenile abalone using a series of experiments. Hatchery produced "button-size " (10 – 12 mm diameter) *H. rubra* were seeded at moderate densities of $100 – 120/m^2$ in a series of small-scale experiments to test the importance of clustered versus more dispersed seeding, where and when they are seeded and whether common black sea urchins shelter abalone from predators. Possible adverse effects of hatchery rearing on shell colour and vigour on survival following release in the wild were also investigated. All experiments were done at Port Stephens and were sampled at daily, weekly, fortnightly, monthly and 2-3 monthly intervals up to 6 months. In all cases apparent survival fell to 7.6 – 30.6 % within the first week and 0.05 – 0.61% after 6 months. The spatial configuration at which abalone were deployed and their proximity to urchins had little effect on their long-term survival.

Program 2

b) The second program culminated in a single large-scale release experiment initiated in July 2005. Juveniles ("button-sized": 10 – 12 mm) housed in miniature predator protective release devices (capsules) and broadcast seeded onto pre-surveyed juvenile habitats during daylight hours from a surface craft at low densities. Seeding from the surface avoided costly deployment by SCUBA divers. The release capsules that were specially developed for these operations each hold only 10-20 button-sized abalone. A key design feature of the capsules is that they pack together to form complete level platforms through which seawater can be continuously passed. This feature together with the use of intensive light enabled in excess of 95% of the abalone to self-load into the capsules. Once loaded, the abalone can be maintained within the capsules in good health for several days of storage awaiting either more favourable sea and weather conditions or road transportation to sites up to several hundred kilometers from the hatchery.

This new deployment technique enabled seeding at densities of 8.6 – $10/m^2$ seed that approximate those previously reported as most common for this size/age class in the wild. This experiment was conducted on a much more expansive spatial scale (1000 m^2 replicate sites as opposed to 1 m^2 used in the first approach. and involved much larger numbers of seed abalone (around 8,600 to 10,000 per replicate site). The survival of abalone will be e valuated in 2009 when it is estimated that seeded abalone will approach legal size. This will give mortality data for use in the bio-economic model (see objective 4) and definitive results to test the hypothesis that abalone populations can be enhanced through seeding. This long-term experiment being conducted on the Tomaree Peninsula, Port Stephens, NSW will also evaluate environmental and genetic implications of seeding large numbers of cultured seed produced from a small number of parent stock on depleted wild populations. Definitive results of this experiment over the next two successive (3 to 4 year) generations will be assessed and reported in due course.

Objective 3 – Development of indigenous community capacity to produce seed abalone for fisheries enhancement, ranching and farming of backlip abalone

As part of the NSW Government's Indigenous Fisheries Strategy, several initiatives were undertaken to develop an indigenous hatchery on the NSW south coast. These initiatives included a generous subsidy offered through an "expression of interest" to indigenous groups with a proposal, business plan and sufficient capacity to establish an effective hatchery. Unfortunately, no suitable proposals were received and the subsidy was not made available. The second initiative was training. One or 2 day technical study tours and 5 day hands-on technical training courses were staged at the abalone aquaculture R&D unit Shoal Bay Port Stephens. These were developed and implemented collaboratively by NSW TAFE and NSW DPI as an initiative under the State government's Indigenous Fisheries Strategy. A total of 11 study tours and 8 one week training courses were staged for coastal indigenous community groups and students enrolled in training courses at the Tomaree and Wollongong colleges of TAFE. For the third initiative, a comprehensive operations manual for high efficiency hatchery and nursery production of blacklip abalone was prepared and published (see under Publications – Appendix 9.3.)

Objective 4 –Development of a bio-economic model as an aid to cost-effective fisheries enhancement of New South Wales blacklip abalone, Haliotis rubra (Leach) fishery

Definitive data for these models will be available when ongoing research (see Part 2b above) is completed in 2 to 3 years time. A separate report on that topic will be provided as soon as practicable after then. A major discussion paper on this topic, that incorporates results to date from this project and from its precursor (FRDC 1998/219) was prepared for the World Congress of Malacology in July 2004 and is included in this report (Chapter 4.4). This discussion paper begins by reviewing the diverse and often complex essential components of successful marine stock enhancement and how these components are being addressed in relation to enhancement of the depleted New South Wales blacklip abalone (*Haliotis rubra)* fishery. Attention is drawn to the need to identify and quantify recruitment and production limiting factors. Key amongst these are age and size specific growth and natural mortality rates and carrying capacity crustose coralline coated rock habitats of surface grazing juveniles. Fisheries production limitations for *H. rubra* also encompass the influence of other species within reef ecosystems. Especially important are predators such as wrasse and other large surface grazing gastropods and urchins. The latter not only compete directly for food (epiphytes and drift seaweed), space and shelter, but may also limit *H. rubra* recruitment by inadvertent grazing on recently settled postlarvae.

KEYWORDS: Abalone, Fishery, Aquaculture, Hatchery, Broodstock, Production, Deployment, Reseeding, Enhancement

1.   BACKGROUND

The NSW abalone fishery is based entirely on *H. rubra* and is largely confined to the southern half of the state. Annual catch (Figure 1) peaked at about 1,200 t in 1971/72, and remained above 600 t through to the early 1980s. Since 1973 a succession of fishery management initiatives (Figure 1) have been implemented. These have included a reduction in effort through license regulation, limiting catch by quota allocation and introduction of size limitations. Regional fishing closures were also imposed between Port Stephens and Jervis Bay (35º 03´S; 150º 44´E) following a major depletion of stocks by the disease *Perkinsus* between 2000 and 2002. A total allowable catch quota of 370 t was first introduced in 1989. Subsequently it has been progressively reduced to 333 t in 1996, to 305 t in 2000, 281 t in 2003, 218 t in 2004 (NSW Fisheries, 2004) and 130 t in 2005 (G. Liggins, pers. comm.).

Many complex questions and issues need to be addressed if this continuing decline in the fishery is to be stopped or reversed. Foremost is the issue of raising juvenile recruitment to levels that match the inherent capacities of individual depleted reefs to sustainably yield higher quantities of marketable 4+ year old adults. Division of the NSW abalone fishery into six geographical management zones (Figure 2) reflects a very steep south to north gradient of decreasing in the abundance of *H. rubra*. Almost 80% of Total Allowable Commercial Catch (TACC) quotas are taken on average from zones 4 to 6 that collectively fall within 150 km of the Victorian border. At the opposite extreme, catches from zone 1, that stretches 600 km north from Jervis Bay to the Queensland border, have fallen from around 20% of total commercial landings in the mid 1980's to only about 5% over the recent years (Anon. 2002). The relative importance of such interactive factors as recruitment, availability of food, (primarily drift seaweed, Shepherd and Hearn (1983)), competition for food and shelter from other benthic herbivores, and differential mortality factors, including natural predation, fishing pressure and disease (especially *Perkinsus*) on this changing pattern of abundance, is not well understood.

Higher sea temperatures in the north are undoubtedly a greater constraint to full development of gonads and hence to successful spawning and recruitment in some years. These processes require at least 1200 degree days at moderate temperatures in the range 8 to 18°C (Grubert and Ritar 2003). Likewise, losses of up to 90% of stocks in the Port Stephens area of zone 1 between 2000 and 2002, attributed to stress-induced susceptibility of *H. rubra* to the disease *Perkinsus*, could, together with impaired breeding, be linked to increased water temperatures. This has also been suggested as a possible cause of the decline of another abalone fishery by Shepherd *et al.* (1998).

During the course of a preceding 3 year project (FRDC 98/219) by Heasman *et al.* (2004), considerable progress was made towards cost-effective enhancement of the fishery through reducing costs of production and transportation of *H. rubra* seed and from the identification of "button size", 6 to 9 month old juveniles as the most appropriate class of seed for enhancing depleted sub-populations. The detection of a major apparent flaw in the deployment of these juveniles in large clusters of 700 to 1500 and development of low density dispersed seeding technology was also a significant step forward. This finding suggested that seeding intensity be aligned with surface grazing based carrying capacity limitations of CCA (crustose coralline algae) rock habitats in relation to juveniles up to at least 12 months old and 25 mm SL.
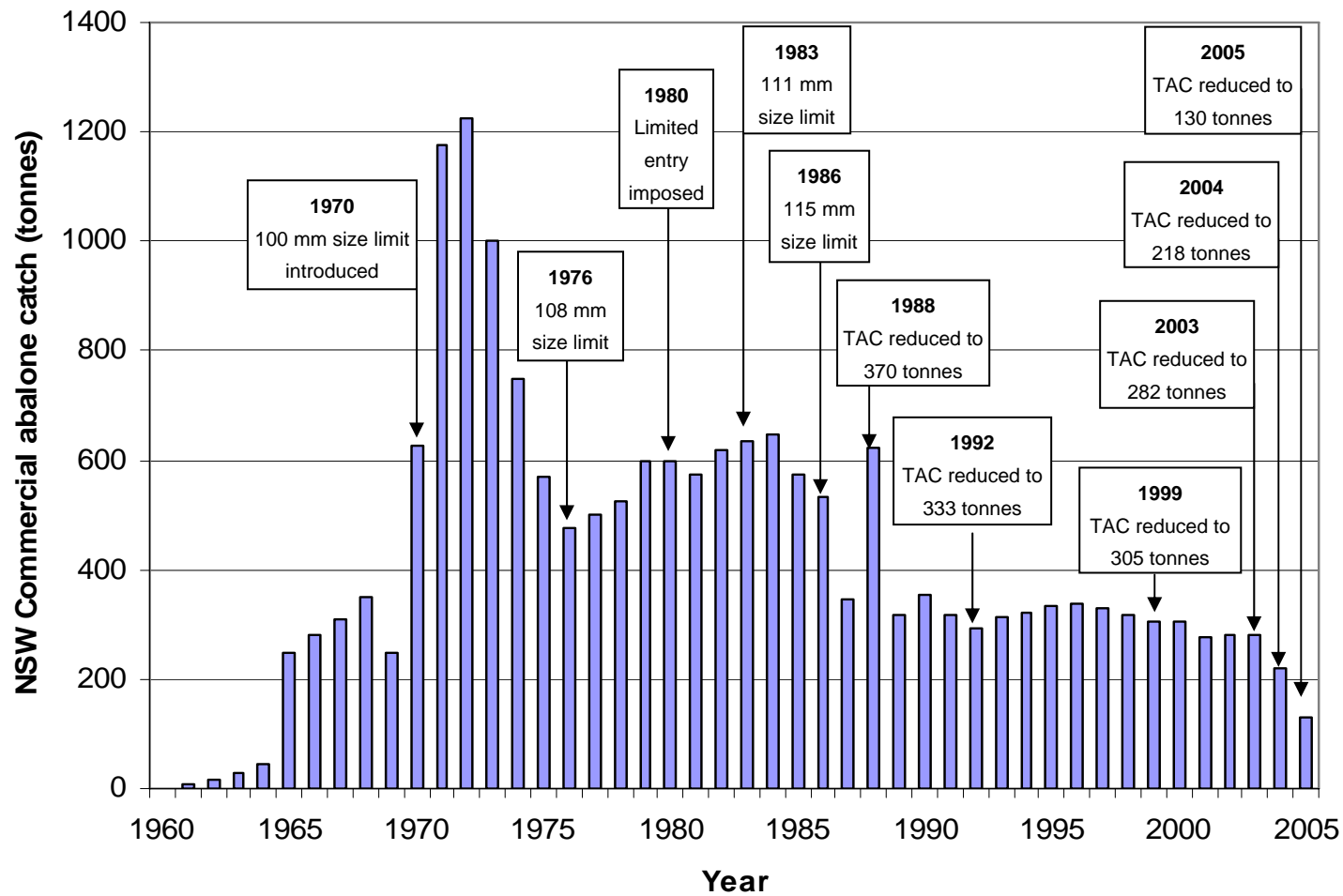
Figure 1. Annual commercial catch data and management initiatives for the NSW *H. rubra* fishery since its inception in 1960. (Source data: Anon 2002; NSW Fisheries 2004, G. Liggins, pers. comm.)
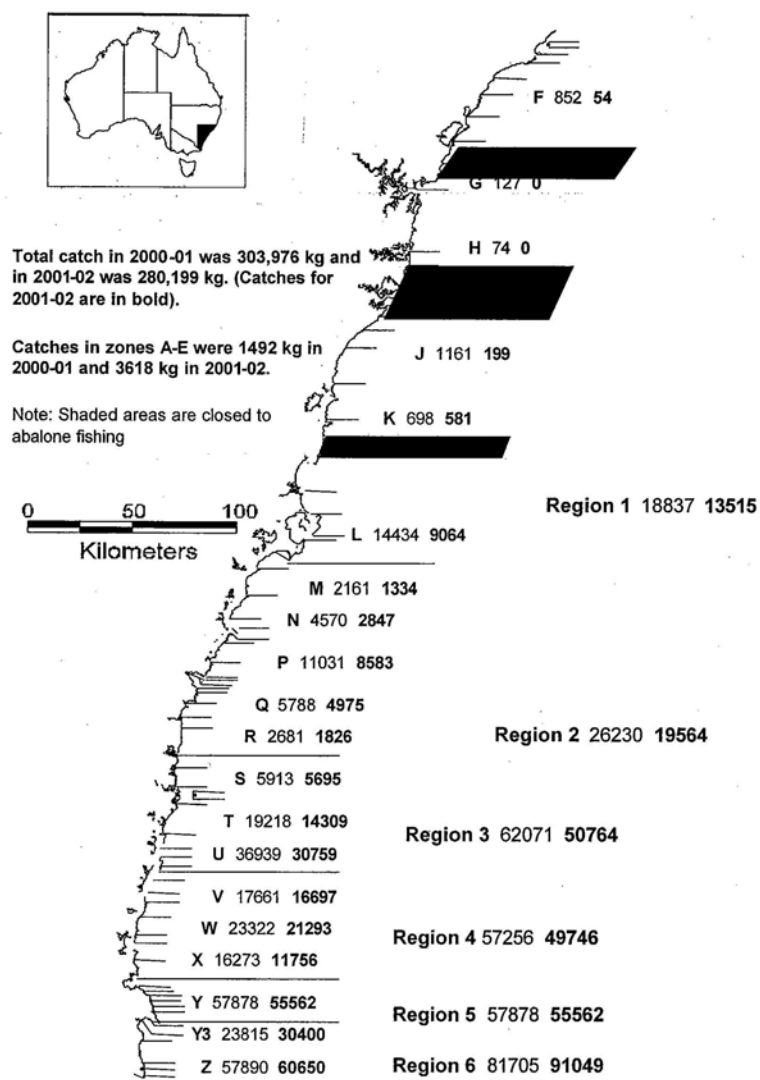
Figure 2. Abalone fishery zones and catches (kg) for financial years 2000-01 and 2001-02 (Anon. 2002*).*

2. NEED

Several critically important steps to achieving one central long-term aim of this project, namely cost-effective enhancement of the now severely depleted *H. rubra* fishery in NSW using hatchery produced seed stock, were achieved during the course of a preceding 3 year project (FRDC 98/219) by Heasman *et al.* (2004).

The first was year-round controlled temperature conditioning and spawning of captive broodstock. This provided impetus for funding and guidance for practical implementation of a complementary R&D project (FRDC 2000/204) to refine and improve this technology in relation to both *H. rubra* and the greenlip abalone *H. laevigata*. This controlled breeding technology has in turn provided a number of important flow-on benefits to the abalone aquaculture industry in southern Australia. Most importantly it is facilitating an industry-wide selective breeding program through facilitated synchronisation of spawning that will enable pair crossing and identification of specific genes for faster growth.

Another important benefit for abalone farmers was development of technology that enables greatly improved nursery production systems at much lower initial capital costs. Uptake of this technology is expected in the next generation of land-based farms that will also need to incorporate controlled reproductive conditioning systems and an additional intermediate nursery system for weaning and on-growing 1 – 3 mm post-larvae. Controlled year-round conditioning and spawning also has the potential to facilitate production and evaluation of triploid abalone especially prospects of faster growth and reproductive sterility, both of which could be of major benefit to future fisheries enhancement and farming of *H. rubra*.

Other significant outcomes in relation to development of cost-effective enhancement of abalone fisheries was that seeding of larval abalone was shown to be fraught with practical difficulties and likely to be cost-prohibitive even if these difficulties could be overcome. Almost a million juvenile *H. rubra* mainly in the "button size" range of 5 to 15 mm were produced and deployed in large clusters of 700 to 2,500 within predator protective release devices. Average growth rates were similar at all sites and similar to that of wild stock with legal size of 115 mm projected to be reached after 4 years. However survival of these seeded juveniles after a year or so was only 0 – 4% and compared poorly with rates of 12 – 40% expected of wild counterparts.

These low survival rates prompted a review of published information on the natural biology of juvenile *H. rubra*. This revealed that natural densities of 12 – 18 month old juveniles on natural rocky reef substrates were consistently low, commonly 2 or less per square metre of reef. This knowledge prompted a final seeding experiment to test the hypothesis that "button size" juveniles should be seeded sparsely to overcome much higher than normal losses experienced so far in this project. Results one year after release supported this hypothesis. Average survival of "button size" juveniles seeded as 10 dispersed groups of 100 was 12.5%. This was within the expected normal range of wild abalone and more than 3 times the average rate of 3.8% for abalone seeded in single clusters of 1,000. This result suggested that continuation of this line of seeding technology R&D was warranted as did predictions of a simple model developed to compare the respective benefits and costs of producing and deploying different age/size classes of seed *H. rubra* from ready-to-set larvae through to advanced (40 – 60 mm) 2 year old juveniles. These predictions strongly suggested that "button size" juveniles offered best prospects of cost-effective seeding.

Practical achievement of cost-effective enhancement of the NSW abalone fishery using hatchery produced seed will nevertheless depend on a wider array of post-release survival issues than just seeding density. Other issues will need to include predation and competition for food and shelter

from other epiphytic and macro algal grazers, particularly urchins, chitons, limpets, turban snails and other gastropods. Other potentially important factors are the health and vigour of juveniles at the point of release. Recent findings of Kemp (2001 and pers. comm.) suggest that use of soya bean meal based formulated diets developed for farmed abalone in Australia may seriously compromise the health and vigour of hatchery produced seed abalone. A linked issue is that formulated diet fed ex-hatchery juveniles are both predator naive and coloured a conspicuous blue-green. By contrast, their cryptically pink to mauve coloured wild counterparts are very difficult to discern within typical crustose coralline coated rock habitats.

An additional seed and sustainability issue is the need to demonstrate that the use of hatchery produced seed to boost sustainable catches will not jeopardise the genetic integrity of existing wild stocks of abalone nor the integrity and diversity of reef communities.

Potential benefits of this continuing research are substantial. For instance, raising of current NSW annual commercial catch to levels of around 600 tonnes, regularly achieved 10 to 15 years ago, would more than increase the current (2007) gross value of the fishery five fold from about $6 million to about $30 million p.a.

This project will also focus on the benefits of reseeding and farming of abalone to indigenous communities on the NSW south coast. These communities have historically relied on abalone as a food source and for cultural purposes. With the significant reduction in both the abundance and availability of abalone this is now denied them. The new technology being developed in this project provides an opportunity to redress this imbalance. Indigenous groups are also seeking sustainable business opportunities for their communities and initiated preliminary studies on establishing hatchery based farming, fisheries enhancement and ranching of abalone in NSW (Heasman, 1993). Although the economic rules applying to any business are the same, some south coast aboriginal groups possess clear advantages over others when it comes to abalone farming and reseeding of abalone. These include current ownership and future additional claims to coastal land and adjacent fisheries resources (2006 ruling by the High Court of Australia in relation to Blue-mud Bay NT) and hence the potential to secure sea ranching rights.

3.   OBJECTIVES

- Produce and assess the utility of sterile triploid blacklip and/or hybrid abalone for fisheries enhancement, ranching and farming.
- Evaluate alternative methods of reducing high post release mortality rates commonly sustained by hatchery produced abalone seed.
- Develop indigenous community capacity to undertake the production of seed abalone and use of that seed for cost-effective and sustainable fisheries enhancement, ranching and farming of back-lip abalone.
- Develop and refine economic models as an aid to developing sustainable and cost-effective fisheries enhancement and ranching of blacklip abalone in NSW.

4.   RESEARCH AND RESULTS

4.1.   Production and assessment of triploid *H. rubra* for farming and fisheries enhancement

This research was structured to address the following logical sequence of tasks and is reported accordingly below:

1. To evaluate and optimise use of cytochalasin B (CB) for initial induction of triploidy in *H. rubra.*
2. To evaluate and optimise use of 6-DMAP for initial induction of triploidy in *H. rubra.*
3. To evaluate intermediate term success of CB and 6-DMAP for induction of triploidy in *H. rubra.*
4. To investigate interactive effects of temperature and ploidy of the relative growth and feeding performance of juvenile *H. rubra.*
5. To assess the practical significance of effects of triploidy on growth and reproductive performance of *H. rubra* for farming and fisheries enhancement.

4.1.1.   Evaluation of cytochalasin B (CB) treatments for triploidy induction in the blacklip abalone, *Haliotis rubra* (Leach)

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4.1.1.1.   Abstract

The effectiveness of cytochalasin B (CB) treatments for inducing triploidy was evaluated in the blacklip abalone *Haliotis rubra* (Leach, 1814) in two orthogonal design experiments. The first experiment employed three dosages (DS) of 0.25, 0.5 and 1.0 mg CB L^{-1}, three starting times (ST) of 5, 15 and 25 min post-fertilization, and three treatment durations (TD) of 10, 20 and 40 min, for a total of 27 treatments. The second experiment compromised of two DSs of 0.25 and 0.5 mg CB L^{-1}, five STs of 5, 15, 20, 25 and 30 min post-fertilization, and three TDs of 10, 20 and 40 min, for a total of 30 treatments. Water temperature was held at $17.5 - 18.5^{o}$C. Day 3 larvae were sampled for triploidy by flow cytometry (FCM), and survival. Optimal inductions were treatments starting at 15 or 20 min post-fertilization and continuing for 40 min, and those initiated 25 or 30 min post-fertilization for 20 or 40 min, using 0.5 mg CB L^{-1}. These treatments were all targeted at inhibition of the second polar body (PB2) formation and yielded triploidy rates of 84.8 – 89.5% coupled with (relative) survival rates of 20.1 – 52.1% in the first experiment, and corresponding rates of 86.5 – 96.5% and 33.0 – 74.1%, respectively, in the second experiment. A common and essential feature of these optimal conditions is that treatment must fully span the period of time for most of the eggs to extrude PB2. Treatments that resulted in suppression of the first polar body (PB1) formation induced triploidy levels below 71.5 and 57.6% in experiments 1 and 2, respectively. Treatments that had overlapping effects on both PB1 and PB2 extrusion led to triploidy rates above 80% but very low survival rates of 1.8 and 5.4% in experiments 1 and 2, respectively.

4.1.1.2.   Introduction

Functional sterility or reduced reproductive output imposed by triploidy may lead to faster growth and better year round marketable products in farmed molluscs (Allen and Downing 1986, 1990; Barber and Mann 1991; Cox *et al.* 1996; Eversole *et al.*, 1996; Hand *et al.* 1998; Ruiz-Verdugo *et al.* 2000). Triploidy in molluscan shellfish can be induced by inhibiting extrusion of the first polar body (PB1) or the second polar body (PB2) through various methods of either chemical or physical treatment (Beaumont and Fairbrother 1991). Suppression of PB2 formation using cytochalasin B (CB) is the most commonly adopted and preferred method with bivalves, although more recently 6-dimethylaminopurine (6-DMAP) has also been used (Desrosiers *et al.* 1993; Gérard *et al.* 1994; Nell *et al.* 1996; Zhang *et al.* 1998; Norris and Preston 2003).

Inhibiting PB1 formation, on the other hand, generally results in both low yields of triploidy and low larval survival (Guo *et al.* 1992a; Gérard *et al.* 1999) and is therefore not commonly practised. Although suppressing both PB1 and PB2 formation is rarely practiced, the production of viable tetraploid spat as achieved by Scarpa, Wada and Komaru (1993) when applying this method to the mussel *Mytilus galloprovincialis* is of interest.

In the present study, the efficiency of CB treatments for inducing triploidy was evaluated in the blacklip abalone *Haliotis rubra* (Leach, 1814). Optimal induction conditions, in terms of CB dosage, starting time of treatment, and treatment duration, were determined for this species.

4.1.1.3. *Materials and methods*

Broodstock, gamete production and fertilization

Two experiments were conducted on separate occasions. Broodstock *H. rubra* were conditioned in a recirculating seawater system at temperatures in the range of $15 - 17^{o}$C (Savva *et al.* 2000). The hydrogen peroxide method of Morse *et al.* (1977), with further modification by Tong *et al.* (1992), was applied to induce spawning.

In the first experiment, first generation (F1) hatchery reared *H. rubra* were used as broodstock. Eggs collected from three females were pooled in a beaker and adjusted to a density of approximately 600 eggs mL^{-1}. Sperm mixed from two males was then added to fertilize the eggs, at a final concentration of approximately 1×10^5 spermatozoa mL^{-1}. Three min after sperm addition, aliquots of 10-mL egg suspensions (~6,000 activated eggs) were dispensed into 140-mL 'mini' down-wellers fitted with 98-μm mesh screens. The down-wellers were kept in clean seawater before treatment was commenced. Seawater was filtered to 1 μm (nominal) by passing a cartridge filter and treated with UV light generated by a 40-W UV lamp. Temperature was kept at $17.5 - 18.5^{o}$C during spawning, fertilization and triploidy induction treatment. The above procedures were repeated in the second experiment, except that wild-caught adult broodstock were used and eggs were collected from four females.

Triploidy induction and larval rearing

In the first experiment, factorial combinations of three dosages (DS) of 0.25, 0.5 and 1.0 mg ⁻ CB L $^{-1}$, three starting times (ST) of 5, 15 and 25 min post-fertilization, and three treatment durations (TD) of 10, 20 and 40 min, were applied to inhibit polar body formation. This resulted in a total of 27 treatments, plus an untreated diploid control. In the second experiment, factorial combinations of two DS's of 0.25 and 0.5 mg CB L $^{-1}$, five ST's of 5, 15, 20, 25 and 30 min post-fertilization, and three TD's of 10, 20 and 40 min, a total of 30 treatments were applied together with a diploid control. In both experiments, three down-weller replicates were applied to each treatment as well as the diploid control. Groups of three down-wellers were strung onto a plastic rod for easy handling and to facilitate accurate timing in the application of various treatments.

From previous observation, *H. rubra* eggs were known to begin to extrude PB1 and PB2 7 – 9 and 28 – 32 min, respectively, after contact with sperm, and first cleavage occurred 75 – 85 min post-fertilization, under a standard hatchery temperature regime of $17 - 19^{o}$C. Therefore, treatments commenced 5 min post-fertilization and applied for 10 or 20 min were intended to inhibit PB1 extrusion. Treatments initiated at the same time but maintained for 40 min were intended to block both PB1 and PB2 release. Those started between 15 and 30 min post-fertilization were intended to suppress PB2 extrusion only (see also Tables 1 and 4).

At each ST, down-wellers were immersed in 1L seawater baths containing appropriate concentrations of CB (Sigma Aldrich, Australia) and 0.1% di-methyl-sulfoxide (DMSO). The eggs were rinsed of residual CB by immersing the down-wellers in seawater containing 0.1% DMSO for 30 min after treatment. The down-wellers were then installed into a flow-through seawater system and provided with an average flow rate of 80 – 120 mL min^{-1} per down-weller and maintained at $16.7 - 18.0^{o}$C throughout the course of incubation and for a further two days of larval rearing. Water levels of the system were adjusted so that each down-weller was operated at a seawater volume of about 100 mL.

Results of preliminary trials (unpublished data) had showed that elevated mortality of *H. rubra* larvae most commonly occurred before the completion of 180° torsion of the foot mass (day 2 larvae). Once this critical morphological change was completed, survival of the larvae appeared to stabilise and was comparable between triploids and diploids (Liu *et al.* 2004). Therefore in the present study, day 3 larvae (around 70 h post-fertilization) were chosen to determine rates of triploidy and survival. By this time, the larvae had already begun to develop eyespots. Triplicate 1-mL samples were taken from each down-weller and fixed in 7% formalin. Microscopic examination of the samples was later used to determine yields of normal looking larvae. The remaining larvae in each down-weller were then sieved onto a 160-μm mesh. Normal looking larvae were retained on the mesh and collected for ploidy assessment while most abnormal larvae were smaller and were eliminated when they passed through the mesh.

Flow cytometry (FCM)

Percent triploidy was verified by FCM, using a modified sample preparation protocols developed for oyster larvae (Allen and Bushek 1992; Eudeline *et al.* 2000). Briefly, several hundred larvae from a treatment replicate were concentrated into a 1.75-mL micro-tube, to which 0.65-mL propidium iodide (PI) staining solution was added. The latter comprised: 2 parts CTX-100 stock; 1 part PI stock; 1 part RNAse stock with 10% of total volume DMSO, (where: CTX-100 stock = 0.1% sodium citrate and 0.1% Triton X-100 (Sigma) in distilled water; PI stock = 1 mg PI mL^{-1} CTX-100 stock and RNAse stock = 1 mg RNAse (Sigma, type 1AS) mL^{-1} distilled water) (Allen 1994). Several micro-tubes were then placed into a larger specimen bottle. The bottle was then subjected to vigorous but standardised (in terms of magnitude, frequency and duration of oscillation) manual shaking regime during which the micro-tubes were bounced rapidly between the lid and floor of the bottle. By this procedure, sufficient numbers of larval cells to apply FCM were dislodged and dissociated into the PI staining solution. In preliminary trials (unpublished data) it was found that eluted samples prepared by this technique can be directly analysed using FCM, transported on ice for a couple of hours, or kept frozen at –80°C for later analysis, with good reproducibility of results. In all the cases, the thawed PI staining solution containing larval cells was collected, aspirated several times through a 1-mL syringe fitted with a 21-gauge needle to disaggregate clustered cells, and filtered through a 35 μm-mesh immediately before being analysed by FCM.

Statistical analysis

A fixed three-way ANOVA model was used to analyse the effects of the three induction factors (DS, ST and TD) and their interactions on percent triploidy and survival, followed by a Fisher's least significant difference (LSD) multiple range test to determine significant differences among means ($P < 0.05$), (Statgraphics Plus for Windows 4.1, Manugistics Inc, USA). Data were square-root transformed, if necessary, to remove heterogeneity of variances as confirmed by Bartlett's test. The diploid control introduced in each experiment was a common control, serving to locate the diploid peaks for flow cytometry analysis and to indicate the egg quality by presenting survival data, rather than for statistical comparison against the treatments.

4.1.1.4.   Results

Early cytology

PB1 and PB2 extrusions were first observed 8 and 32 minutes post-fertilization, respectively, in experiment 1, and 9 and 32 minutes, respectively, in experiment 2. First cleavage was first observed 82 and 85 min. after fertilization in experiments 1 and 2, respectively.

Experiment 1

Triploidy induction

Percent triploidy in day 3 normal larvae varied between 24.7 and 90.1% across the 27 treatments (Table 1). No obvious triploid peaks were detected in the diploid control.

Due to the poor overall relative larval survival rates of 0.9 – 15.5% and obvious aneuploid peaks in treatments associated with the use of 1.0 mg CB L^{-1} (treatments 19 – 27, Table 1), triploidy and survival from all these nine treatments were excluded from the statistical analysis. It should be noted that the absolute survival rate in the diploid control was also low (13.3%) (see Experiment 1 – *Survival*). Three-way ANOVA on the remaining data (treatments 1 – 18, Table 1) showed that DS, ST and TD all had highly significant effects ($P \leq 0.0001$), as did all their interactions, on percent triploidy (Table 2).

Treatments started 5 or 15 min post-fertilization and continued for 40 min, and treatments started 25 min post-fertilization for 20 or 40 min, using either 0.25 or 0.5 mg CB L^{-1} (treatments 3, 6, 8, 9, 12, 15, 17 and 18, Table 1), induced high rates of triploidy of $\geq 80.7\%$ by inhibiting PB2 extrusion (except for treatment 12). Although these high rates of triploidy were not uniform, they were significantly higher ($P < 0.05$) than those of all other treatments (Table 1).

Treatments started 5 min post-fertilization and applied for 10 or 20 min. were designed to inhibit PB1 extrusion. However, a dosage of 0.25 mg CB L^{-1} was not able to block PB1 formation sufficiently for the low triploidy rates of 24.7 and 28.6% obtained (and the comparable survival rates to the diploid control) (treatments 1 and 2, Table 1). The use of 0.5 mg CB L^{-1} (treatments 10 and 11, Table 1) resulted in triploidy rates of only 52.3 and 71.5%, which were substantially lower ($P < 0.05$) than those best triploidy rates achieved by inhibiting PB2 extrusion.

Treatments commenced 5 min post-fertilization and continued for 40 min. were aimed at suppressing extrusion of both PB1 and PB2. However using 0.25 mg CB L^{-1} as in treatment 3 (Table 1), failed to inhibit PB1 extrusion in the first 20 min. of treatment (as indicated by results of treatments 1 and 2, Table 1). It is therefore deduced that the triploidy rate of 83.8% returned by treatment 3 was due to the retention of PB2 in the second 20 min of exposure to 0.25% CB. The treatment that applied CB at 0.5 mg L^{-1} (treatment 12, Table 1) actually suppressed both PB1 and PB2 formation, resulting in a high triploidy rate of 80.7%, but with the lowest survival rate of 0.2% attained across all the treatments using either 0.25 or 0.5 mg CB L^{-1} in this experiment.

Survival

Yields of normal day 3 larvae across the 27 CB treatments were consistently low, ranging from 0.1 to 13.6%. As mean yield of larvae from the diploid control was similar at 13.3% these universally low absolute survival rates are attributed to the poor quality of eggs spawned by the recently matured F1 broodstock (the first spawning in their life) or some other extraneous factor, Additional evidence of the poor egg quality were observations that newly spawned eggs did not have a well-round shape nor uniformly dense yolk and that frequencies of abnormal larvae and non viable eggs (unpublished date) were unusually high. Nevertheless, survival rates relative to the control treatment of 0.9 – 102.4% (Table 1) still provided a valuable guide for optimising triploidy induction protocols.

A three-way ANOVA analysis of relative survival data from which results of treatments using 1.0 mg CB L^{-1} had been omitted, revealed highly significant effects ($P \leq 0.0002$) of DS and of interaction between DS and ST on survival. The effects of ST and TD were also significant ($P < 0.05$). However, interactions between DS and TD, between ST and TD, and among DS, ST and TD, had no significant effects ($P > 0.05$) on survival (Table 3).

Experiment 2

Triploidy induction

Rates of triploidy obtained in day 3 larvae ranged between 13.3 and 94.6% across the 30 treatments (Table 4). As in experiment 1, no obvious triploid peaks were detected in the diploid control.

Three-way ANOVA also indicated that percent triploidy (after square-root transformation) was highly affected (($P \leq 0.0001$) by DS, ST and TD, and all their interactions (Table 5). In contrast to experiment 1, where triploidy rates up to 88.6% were recorded in treatments using CB at 0.25 mg L^{-1} (Table 1). Corresponding rates of induced triploidy in this experiment were $\leq 46.2\%$ (Table 4). However, within these low triploidy yields, there was still considerable variation ($P < 0.05$) between treatments (data not shown), with results following the same general trends of variation as in experiment 1. For example, treatments started 5, 15 or 25 min post-fertilization and continued for 40 min (treatments 3, 6 and 12, Table 4) induced the highest level of triploidy at this CB concentration.

For treatments using 0.5 mg CB L^{-1}, those initiated 5, 15 or 20 min post-fertilization and continued for 40 min (treatments 18, 21 and 24, Table 4); those commenced 25 min post-fertilization and maintained for 20 or 40 min (treatments 26 and 27, Table 4); and those started 30 min post-fertilization and lasted for either 10, 20 or 30 min (treatments 28, 29 and 30, Table 4), yielded high rates of triploidy $\geq 80.2\%$ (See also Figure 1). All these treatments were targeted at inhibiting PB2 extrusion, except for treatment 18. Although induced triploidy rates within this group of treatments varied considerably, they were significantly different ($P < 0.05$) from those of other treatments. The only exception was a triploidy rate of 80.2% in treatment 28 (Table 4), which was not significantly different ($P > 0.05$) from that of 74.8% in treatment 23 that was initiated 20 min post-fertilization and applied for 20 min.

Inhibiting PB1 extrusion using 0.5 mg CB L^{-1} as in treatments 16 and 17 (Table 4) induced triploidy rates of 33.1 and 57.6% that were significantly lower ($P < 0.05$) than best triploidy rates achieved with treatments targeted at inhibiting PB2 extrusion. Targeting inhibition of both PB1 and PB2 extrusion however, resulted in high triploidy yields of 88.0% (treatment 18, Table 4) albeit with the lowest survival rate of 2.2% among all 30 treatments in this experiment.

Survival

Larval survival across the 30 treatments ranged from 2.2 to 40.0%, while that in the diploid control was 40.7%. The corresponding range of relative survival rate was 5.4 to 98.3% (Table 4).

Three-way ANOVA found highly significant effects ($P \leq 0.0079$) on survival of DS, ST, and interactions between DS and ST, and between ST and TD. The effect of interaction of DS, ST and TD was also significant ($P < 0.05$) (Table 6).

4.1.1.5.   Discussion

Optimization of induction conditions

Experimental treatments that yielded both high rates of triploidy and acceptable rates of survival in day 3 *H. rubra* larvae are summarized in Table 7. In both the experiments, treatments initiated 15 min post-fertilization and continued for 40 min, and treatments initiated 25 min post-fertilization and applied for 20 or 40 min, using 0.5 mg CB L^{-1}, appeared to be best. Moreover, treatment initiated 20 min post-fertilization and applied for 40 min, and treatments initiated 30 min post-fertilization and continued for 20 or 40 min were also effective in the second experiment. These optimal conditions yielded high triploidy rates of 84.8 – 89.5% with relative survival rates of 20.1 –

52.1% in day 3 *H. rubra* larvae in the first experiment. Likewise, high triploidy rates of 86.5 – 96.5% accompanied by relative survival rates of 33.0 – 74.1% were achieved in the second experiment, though significant differences ($P < 0.05$) occurred between rates of triploidy or survival in some treatments in experiment 2. All these optimal treatments were targeted at blocking PB2 extrusion.

FCM and experimental system

Abalone larvae are lecithotrophic (non-feeding) and possess large amounts of yolk as the main energy reserves for their early ontogeny. During the early stages of this study it was found that when *H. rubra* trochophores or early stage veliger larvae were subjected to FCM, the resultant traces lacked any detectable peaks and could not be properly interpreted. The underlying reasons are not understood, but could have been associated with interference of DNA/RNA fragments attached to tissue fragments and emulsified yolk granules released during the maceration of the. Such particles may outnumber the targeted tissue cells thereby rendering ploidy peaks. The choice of day 3 larvae for triploidy determination appears to be important for the successful application of FCM, possibly because by this stage the larvae may have utilized a large proportion of yolk reserves. The larval agitation method developed and applied in the present study probably strips off ectodermal cells, does not macerate larvae and therefore does not disturb the yolk mass thereby may also avoiding or minimizing the generation of extraneous debris.

Use of an experimental 'mini' down-weller system facilitated easy handling and accurate timing for the application of treatments. This enabled replicated experiments in which a wide range of induction conditions could be simultaneously applied to a common pool of eggs, and hence examination of treatment effects (DS, ST and TD, and their interactions). Since most previous studies either have not employed replication, or have used different batches of eggs as a means of replication, treatment effects were confounded by 'inter batch' factors especially variable egg quality.

Triploidy induction (by inhibiting PB2 extrusion)

In the present study, optimal conditions for triploidy induction in *H. rubra* were identified (Table 7). Results demonstrate the high efficacy of CB in inducing triploidy in *H. rubra*. In contrast, Yang *et al.* (1998) and Stepto and Cook (1998) using CB were only able to induce triploidy rates as high as 18.9 and 70.9% in *Haliotis diversicolour supertexta* and *H. midae*, respectively.

The optimal concentration of 0.5 mg CB L^{-1} identified here for triploidy induction in *H. rubra* is lower than that of 1.0 mg CB L^{-1} for Pacific oyster *Crassostrea gigas* (Downing and Allen 1987), and 0.75 – 1.5 mg CB L^{-1} for Sydney rock oyster *Saccostrea commercialis* (Nell *et al.* 1996). However, this dosage falls within the range found as suitable for other abalone species, such as 0.25 – 0.5 mg CB L^{-1} for *H. diversicolour supertexta* (Yang *et al.* 1998) and *H. midae* (Stepto and Cook 1998), and 0.5 – 1.0 mg CB L^{-1} for *H. rufescens* (Maldonado *et al.* 2001).

Although a dosage of 0.25 mg CB L^{-1} was able to induce high rates of triploidy ($\geq 80.7\%$) in the first experiment (Table 1), this did not occur in the second experiment ($\leq 46.2\%$) (Table 4). Therefore this concentration is not considered reliable enough for triploidy induction in *H. rubra*. Yields of triploidy obtained in the second experiment did nevertheless follow the same general trend of variation as in the first experiment. These results suggests that *H. rubra* eggs of differing origins, in this case, a hatchery derived F1 generation (experiment 1) and wild-caught adults (experiment 2) may have different susceptibilities to CB treatment, but only if CB is applied at low concentrations, in this case 0.25 mg L^{-1}. A dosage of 1.0 mg CB L^{-1} could also induce high rates of triploidy (Table 1). However, its use was found to result in low relative survival rates in the range of 0.9 – 15.5%. Extended exposure time (40 minutes) at this dosage generated aneuploids

characterized by emission peaks intermediate to triploid and diploid peaks on the FCM histogram traces. The practical implication of these results is that 1.0 mg CB L^{-1} is toxic to *H. rubra* eggs.

The more usual criterion for starting time, as opposed to lapsed time post-fertilization used in the present study, is the time when about 50% of the eggs are observed (microscopically) to be extruding PB1. This criterion was originally recommended by Allen *et al.* (1989) for commercial production of triploid Pacific oyster *Crassostrea. gigas* using CB. Indeed, it provides a valuable basic guideline and has been widely adopted as the time to initiate treatment for chemical induction of triploidy in other bivalves (Barber *et al.* 1992; Nell *et al.* 1996; Supan *et al.* 2000; Ruiz-Verdugo *et al.* 2001) and abalone (Zhang *et al.* 1998; Maldonado *et al.* 2001), regardless of whether CB or 6-DMAP is used. In practice however, we were unable to apply this criterion to *H. rubra*, due to the fact that scanning the periphery of much larger (190 μm in diameter) fertilized eggs of abalone for polar bodies is much more difficult and protracted than for 60 μm diameter eggs of oysters. So much so that PB1 observable in any single plane of view represent 30% or less actual frequencies (unpublished data).

Results achieved in the two experiments of the present study on optimal treatment conditions were in good agreement and indicated that elapsed time post-fertilization could be reliably used to optimise triploidy induction success in *H. rubra* when used in conjunction with a dosage of 0.5 mg CB L^{-1} (Table 7).

The kinetic curves for PB1 and PB2 extrusion and for first cleavage are basically parallel as has been demonstrated in several bivalves (Desrosiers *et al.* 1993; Gérard *et al.* 1994, 1999) (Figure 2 A). This indicates that the development rates of the three sequential events are uniform. Typically, 80 – 100% of *H. rubra* embryos incubated at 17 – 19°C develop to the two-cell stage within ten minutes of each other (unpublished data). It is therefore deduced that within batches of *H. rubra* zygotes, extrusions of PB1 and PB2 are also completed within a time span of less than 15 min. Thus in spite of an impaired ability to directly quantify the progress of polar body extrusion, simple kinetic curves of PB1 and PB2 extrusion for *H. rubra* can be back extrapolated using two lines parallel to that of first cleavage, with origins designated by the times at which PB1 and PB2 were first observed (Figure 2 B). In the present study, the duration of complete extrusion of PB1 and PB2 across batches of *H. rubra* zygotes was in the range of 8 – 23, and 32 – 47 min post-fertilization, respectively. It thus appears that *H. rubra* possesses a different early cytology to that of the Pacific oyster *C. gigas*, being characterized by non-overlapping periods for the extrusion of PB1 and PB2, longer time intervals between PB1 and PB2 formation (almost double that of *C. gigas*) and between PB2 formation and first cleavage, and faster developmental rates (steeper kinetic curves) for all three sequential processes.

CB is an inhibitor of cytokinesis not karyokinesis. In effect, CB treatment is a developmental impass, past which cytokinesis cannot proceed (Downing and Allen 1987). Thus in the context of the proposed early cytology of *H. rubra* eggs (Figure 2 B), CB treatment can be started from 15 to 30 min post-fertilization to inhibit PB2 formation (although treatment started 15 min post-fertilization or sooner may interfere with meiosis I). This is a period that begins after PB1 but before PB2 formation in a population of eggs, and which coincides with chromosome segregation during meiosis II (Guo *et al.* 1992b; Désilets *et al.* 1995), Provided that there are no apparent inhibitory effects on the development (chromosome segregation) of the eggs (Guo *et al.* 1992b), CB does not cause direct toxic harm. It is therefore apparent to ensure high yields of triploidy, CB treatment must span a period of about 32 – 47 min post-fertilization thereby inhibiting most PB2 release (inhibit cytokinesis) within large batches of *H. rubra* eggs. In fact, treatment could possibly be extended to about 75 min post-fertilization without interfering with first cleavage.

These predictions closely agree with results of the present study, in which the highest rates of triploidy were achieved in treatments that ended after 45 min post-fertilization (Tables 1, 4 and 7;

Figure 1). Hence, a simple formula, namely, ST + TD ≥45 min post-fertilization, where ST falls between 15 and 30 min post-fertilization, with TD to be calculated by difference (not to determine TD first), can be used to predict optimal conditions for triploidy induction in *H. rubra*, using 0.5 mg CB L^{-1} at 18 ± 0.5^{o}C. These flexible combinations of ST and TD span the entire time interval needed for most eggs to extrude PB2. This key factor explains the highly significant effects (P≤ 0.0001) of interactions among DS, ST and TD on rates of triploidy (Tables 2 and 5).

As PB2's were extruded up to 28 min post-fertilization it is preferable not to start treatment until 30 min post fertilization. Likewise to negate potential toxicity of CB associated with longer exposure times, TD's should not exceed 40 min. It is important to realize that CB treatment itself may be lethal if a treatment either begins too late or does not fully span the active events for PB2 extrusion across a batch of eggs. This is because CB treatment may disrupt the coordination of karyokinesis and cytokinesis during meiosis by inhibiting only one of the two processes (Downing and Allen 1987; Desrosiers *et al.* 1993). This might explain the low survival rate of 5.5% in the treatment started 30 min post-fertilization and continued for 10 min (treatment 28, Table 4), and may also account for the variation in survival rates in the present study along with the potential toxicity of CB. Regardless, fertilization should be implemented with precision in order to achieve maximum synchrony in the development of the eggs and to reduce inter batch variation.

Downing and Allen (1987) used a pre-determined treatment duration of 15 min to optimise triploidy induction in Pacific oyster *C. gigas*, and found that the best time to start treatment was when about 50% of the eggs had released PB1. Nell *et al.* (1996) obtained similar results also by applying a fixed treatment duration in relation to the induction of triploidy in the Sydney rock oyster *S. commercialis (=glomerate)*. Had a fixed treatment duration of 20 min been employed for *H. rubra* in the present study, optimal starting time according to the above equation proposed for optimal induction conditions, would have been narrowed to 25 – 30 min post-fertilization. For species with an early cytological development sequence similar to the Pacific oyster *C. gigas*, use of 50% of PB1 extrusion for initiation of treatment (using CB) is appropriate. This is because of the relatively short time intervals between the initiation of PB1 and PB2 extrusion, and between PB2 formation and first cleavage (Figure 2 A), which severely constrain both starting time and treatment duration, and could also blur any clear distinction between meiotic I and meiotic II triploids (Gérard *et al.* 1999).

An important finding of this study is that it is inappropriate to assume that 50% of PB1 extrusion is also the best criterion for the triploidy induction starting time of species with early cytology fundamentally different from oysters, or when using alternative chemical inducers to CB such as 6-DMAP. Accordingly, it is likely that best triploidy rates of 57 – 73% in Sydney rock oyster *S. glomerata (=commercialis)* (Nell *et al.* 1996) and 56% in Pacific abalone *H. discus hannai* (Zhang *et al.* 1998) obtained with the application of this criterion could be improved. The development of 50% of PB1 extrusion as a benchmark to begin treatment was in part to accommodate high variability in meiotic rates within between batches of gametes stripped from oysters, a factor that may enormously affect yields of triploids (Downing and Allen 1987; Allen and Bushek 1992). For instance, the time for 50% PB1 extrusion of eggs stripped from the oyster *Crassostrea virginica* was found to vary greatly from 17 to 34 min post-fertilization at 27°C by Allen and Bushek (1992) and from 24 to 31 min post-fertilization at 28°C by Supan *et al.* (2000). With induced spawning however, there is much less variability in the speed of development of the fertilized eggs, For example PB1 occurs in 17 – 19 min post-fertilization at 25°C in Sydney rock oyster *S. commercialis* (Nell *et al.* 1996) and 20 – 22 min post-fertilization at 23°C in Pacific abalone *H. discus hannai* (Zhang *et al.* 1998). Unfortunately, few studies have addressed the importance of appropriate treatment duration that should also vary with meiotic rates (Supan *et al.* 2000).

The very rapid onset and completion of polar body extrusion renders use of 50% of PB1 extrusion as a cue to triploidy induction treatment initiation, unreliable. A small error of just 5 min around

the brief event of 50% of PB1 extrusion may greatly affect triploidy yields even among sub-samples of the same batch of eggs, under a supposedly fixed treatment conditions. The magnitude of this problem is illustrated by large differences in triploidy rates obtained with treatments 20 and 23 (51.7 and 74.8%, respectively, Table 4) and with treatments 23 and 26 (74.8 and 94.6%, respectively, Table 4) of the second experiment. This issue raises the question of what factors actually account for the inter-batch differences in yields of triploids (Maldonado *et al.* 2001; Ruiz-Verdugo *et al.* 2001). High triploidy yields are dependent to some degree on the survival of residual diploids that can be minimized by adoption of treatments that neither start too late nor end too early, using effective CB concentrations. A basic knowledge of the kinetics of early cytology of the potential species targeted will facilitate optimisation of triploidy induction.

Triploidy induction (by inhibiting PB1 or both PB1 and PB2 extrusion)

The highest rates of triploidy that were induced by inhibiting PB1 extrusion were only 71.5 and 57.6% in experiments 1 and 2, respectively, using 0.5 mg CB L^{-1} (treatment 11, Table 1; treatment 17, Table 4). These results were poor compared to those achieved by blocking PB2 extrusion. Though considerable effort was made to produce tetraploid *H. rubra* by inhibiting first cleavage, we failed to detect any pronounced tetraploid peaks in the resultant day 2 – 7 larvae using FCM. On the other hand, future attempts to suppress PB1 extrusion will be made in response to the encouraging observation of small but distinct tetraploid peaks in some samples analysed (unpublished data). Production of tetraploids through suppression of PB1 formation has been reported in other molluscan shellfish (Stephens and Downing 1988; Zhang *et al.* 2000). Low rates of triploidy and apparent production of tetraploids obtained in the present study with *H. rubra* may have arisen from different patterns of chromosome segregation in meiosis II following inhibition of PB1 (Guo *et al.* 1992b).

In theory, retention of both PB1 and PB2 would lead to pentaploidy (Beaumont and Fairbrother 1991). However, the application of this method to mussel *M. galloprovincialis* (Scarpa *et al.* 1993) was also able to produce viable tetraploid spat. Although the exact mechanism of how these tetraploids were induced is not yet well understood, results confirmed that pentaploids were not viable (Cooper and Guo 1989). In the present study, treatments commenced 5 min post-fertilization and applied for 40 min were aimed at suppressing both PB1 and PB2 formation. Although such treatments with a dosage of 0.25 mg CB L^{-1} failed to inhibit PB1 extrusion, they were still able to block PB2 formation (treatment 3, Tables 1 and 4, respectively). This suggests that PB1 extrusion is more resistant to CB treatment than PB2. On the other hand, such treatments using 0.5 mg CB L^{-1} (treatment 12, Table 1; treatment 18, Table 4) were able to inhibit both PB1 and PB2 extrusion. This in turn explains the very poor survival rates of 0.2 and 2.2% that resulted from these two treatments, namely, the resultant pentaploids were not viable. The high rates of triploidy (> 80%) exhibited by the few survivors of these two treatments is unlikely to have resulted from suppressing both PB1 and PB2 extrusion, but the most likely explanation is that it reflected inhibition of one single polar body in a limited number of eggs in asynchronous development.

In conclusion, CB was highly effective in inducing triploidy in *H. rubra*. Optimal conditions are a flexible combination of starting time and treatment duration, targeting inhibition of PB2 extrusion. A common and essential feature of these optimal conditions is that treatment must fully span the entire time interval needed for a population of eggs to extrude PB2. Suppression of PB1 extrusion induced lower rates of triploidy, while suppression of both PB1 and PB2 extrusion led to very poor larval survival albeit high rates of triploidy in the few survivors. Since early cytology and speed of meiosis may vary greatly between species (Desrosiers *et al.* 1993) and with environmental factors such as temperature and salinity (Downing and Allen 1987; Desrosiers *et al.* 1996), it is not valid to extrapolate induction conditions specifically developed for one species to the others, or even to the same species under alternative environmental conditions.

4.1.1.6. References

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Table 1. Rates of triploidy and survival (means ± SD, *n* = 3) in day 3 *H. rubra* larvae at different treatment combinations of CB dosage (DS), starting time (ST) and treatment duration (TD), and results of Fisher's LSD multiple range tests for triploidy and survival in experiment 1. Treatments were targeted at inhibition of PB2 formation unless otherwise addressed.

Treatments	DS (mg L^{-1})	ST (min post-fertilization)	TD (min)	Triploidy (%)	Survival [1] (%)	Relative survival [2] (%)
1[3]		5	10	24.7 ± 1.2	8.9 ± 3.3	66.7
2[3]			20	28.6 ± 1.9	10.2 ± 5.5	76.8
3[4]			40	83.8 ± 6.8	6.3 ± 3.9	47.5
4		15	10	42.6 ± 6.4	8.6 ± 3.8	64.9
5	0.25		20	46.5 ± 1.6	13.6 ± 4.0	102.4
6			40	88.6 ± 3.7	10.5 ± 3.3	78.6
7		25	10	32.9 ± 1.0	3.9 ± 1.7	29.3
8			20	82.8 ± 2.7	3.0 ± 1.2	22.9
9			40	83.2 ± 3.3	5.5 ± 1.0	41.1
10[3]		5	10	53.2 ± 4.2	4.0 ± 3.2	30.2
11[3]			20	71.5 ± 4.4	4.0 ± 1.9	30.2
12[4]			40	80.7 ± 0.8	0.2 ± 0.2	1.8
13		15	10	39.6 ± 3.3	5.1 ± 2.5	38.4
14	0.5		20	57.2 ± 1.2	5.6 ± 1.7	42.1
15			40	89.5 ± 4.3	2.7 ± 1.5	20.1
16		25	10	47.3 ± 5.4	8.4 ± 1.3	63.1
17			20	85.3 ± 4.7	6.9 ± 3.8	52.1
18			40	84.8 ± 1.9	3.5 ± 2.6	26.5
19[3]		5	10	78.7 ± 5.4	0.4 ± 0.4	2.7
20[3]			20	78.6 ± 1.0	0.6 ± 0.4	4.6
21[4]			40	Aneuploid peak[5]	0.1 ± 0.1	0.9
22		15	10	46.3 ± 8.5	2.1 ± 0.6	15.5
23	1.0		20	90.1 ± 0.0	1.1 ± 0.7	8.2
24			40	Aneuploid peak[5]	0.6 ± 0.2	4.4
25		25	10	71.4 ± 20.5	0.6 ± 0.2	4.6
26			20	88.7[5]	0.2 ± 0.2	1.8
27			40	Aneuploid peak[5]	0.1 ± 0.1	1.1
Diploid control				0	13.3 ± 2.3	100

Results of Fisher's LSD multiple range test for triploidy[6]

Treatments	15	6	17	18	3	9	8	12
Triploidy (%)	89.5	88.6	85.3	84.8	83.8	83.2	82.7	80.7

Results of Fisher's LSD multiple range test for survival[7]

Treatments	6	17	3	9	18	8	15	12
Survival (%)	10.5	6.9	6.3	5.5	3.5	3.0	2.7	0.2

[1] Survival = yields of normal day 3 larvae from eggs.

[2] Relative survival = (survival of treatments/the diploid control) × 100.

[3] Treatments targeted at inhibition of PB1 formation.

[4] Treatments attempted to inhibit both PB1 and PB2 formation.

[5] Larvae of the three replicates were pooled due to low survival.

[6] Treatments 19 – 27 were not included in the analysis. Triploidy rates were arranged in a descending order from left to right. Means sharing a same horizontal bar did not differ significantly ($P > 0.05$). Triploidy rates below 80.7% were significantly lower ($P < 0.05$) than any of those listed and were not shown.

[7] Treatments 19 – 27 were not included in the analysis. Survival was arranged in a descending order from left to right. Only survival from the above eight treatments with high triploidy rates was shown. Means sharing a same horizontal bar did not differ significantly ($P > 0.05$).

Table 2.   Three-way ANOVA table for triploidy in day 3 *H. rubra* larvae in experiment 1.

Sources	Sum of squares	DF	Mean squares	F-*ratios*	P-*values*
DS	1,494.68	1	1,494.68	107.31	0.0000
ST	1,470.6	2	735.301	52.79	0.0000
TD	18,381.8	2	9,190.9	659.84	0.0000
DS × ST	991.441	2	495.721	35.59	0.0000
DS × TD	851.018	2	425.509	30.55	0.0000
ST × TD	3,082.93	3	770.733	55.33	0.0000
DS × ST × TD	1,102.59	3	275.649	19.79	0.0000
Residual	501.44	36	13.929		

DS: CB dosage; ST: starting time; TD: treatment duration. Treatments using 1.0 mg CB L^{-1} (treatments 19 – 27, Table 1) were not included in the analysis.

Table 3.   Three-way ANOVA table for survival in day 3 *H. rubra* larvae in experiment 1.

Sources	Sum of squares	DF	Mean squares	*F*-ratios	*P*-values
DS	150.667	1	150.667	17.74	0.0002
ST	62.601	2	31.301	3.68	0.0350
TD	56.73	2	28.365	3.34	0.0468
DS × ST	204.489	2	102.245	12.04	0.0001
DS × TD	35.312	2	17.656	2.08	0.1399
ST × TD	35.582	4	8.896	1.05	0.3966
DS × ST × TD	23.192	4	5.798	0.68	0.6087
Residual	305.827	36	8.495		

ST: starting time; DS: CB dosage; TD: treatment duration. Treatments using 1.0 mg CB L^{-1} (treatments 19 – 27, Table 1) were not included in the analysis.

Table 4. Rates of triploidy and survival (means ± SD, n = 3) in day 3 *H. rubra* larvae at different treatment combinations of CB dosage (DS), starting time (ST) and treatment duration (TD), and results of Fisher's LSD multiple range tests for triploidy and survival in experiment 2. Treatments were targeted at inhibition of PB2 formation unless otherwise addressed.

Treatments	DS (mg L^{-1})	ST (min post-fertilization)	TD (min)	Triploidy (%)	Survival[1] (%)	Relative survival[2] (%)
1[3]		5	10	15.3 ± 0.2	36.1 ± 7.1	88.6
2[3]			20	15.9 ± 0.8	40.0 ± 3.3	98.3
3[4]			40	42.9 ± 4.6	27.7 ± 4.1	68.2
4		15	10	13.3 ± 1.1	31.0 ± 10.0	76.0
5			20	28.2 ± 3.6	33.6 ± 12.0	82.5
6			40	46.2 ± 2.7	37.4 ± 2.0	91.8
7		20	10	14.6 ± 1.5	21.6 ± 7.0	53.1
8	0.25		20	26.5 ± 4.4	20.7 ± 12.2	50.9
9			40	39.7 ± 6.2	19.1 ± 4.3	47.0
10		25	10	19.5 ± 0.7	11.5 ± 7.0	28.3
11			20	30.2 ± 5.5	24.7 ± 8.7	60.6
12			40	43.1 ± 2.7	22.5 ± 8.1	55.2
13		30	10	29.5 ± 2.0	17.4 ± 6.2	42.7
14			20	38.7 ± 1.4	14.0 ± 1.9	34.4
15			40	39.4 ± 3.9	18.0 ± 4.3	44.1
16[3]		5	10	33.1 ± 3.3	10.8 ± 4.4	26.5
17[3]			20	57.6 ± 5.4	10.7 ± 4.3	26.2
18[4]			40	88.0 ± 2.1	2.2 ± 1.9	5.4
19		15	10	17.8 ± 1.1	20.7 ± 12.0	50.9
20			20	51.7 ± 5.5	14.3 ± 1.3	35.2
21			40	96.5 ± 0.5	22.6 ± 9.1	55.6
22		20	10	15.0 ± 0.9	39.7 ± 11.4	97.6
23	0.5		20	74.8 ± 6.1	30.1 ± 11.1	73.9
24			40	92.2 ± 5.7	18.5 ± 6.5	45.3
25		25	10	57.5 ± 4.0	25.9 ± 7.1	63.6
26			20	94.6 ± 0.6	30.2 ± 4.6	74.1
27			40	87.1 ± 5.6	13.5 ± 3.1	33.0
28		30	10	80.2 ± 1.7	5.5 ± 3.4	13.6
29			20	86.5 ± 2.9	26.0 ± 4.7	64.0
30			40	88.8 ± 1.8	17.7 ± 4.3	43.4
Diploid control				0	40.7 ± 2.7	100

Results of Fisher's LSD multiple range test for triploidy (after square-root transformation)[5]

Treatments	21	26	24	30	18	27	29	28
Triploidy (%)	96.5	94.6	92.2	88.8	88.0	87.1	86.5	80.2

Results of Fisher's LSD multiple range test for survival[6]

Treatments	26	29	21	24	30	27	28	18
Survival (%)	30.2	26.0	22.6	18.5	17.7	13.5	5.5	2.2

[1] Survival = yields of normal day 3 larvae from eggs.

[2] Relative survival = (survival of treatments/the diploid control) × 100.

[3] Treatments targeted at inhibition of PB1 formation.

[4] Treatments attempted to inhibit both PB1 and PB1 formation.

[5] Triploidy rates were arranged in a descending order (in the original form) from left to right. Means sharing a same horizontal bar did not differ significantly (P > 0.05). Triploidy rates below 80.2% were significantly lower (P < 0.05) than any of those listed (except for a triploidy rate of 74.8% in treatment 23, which was not significantly different from that of 80.2%) and were not shown.

[6] Survival was arranged in a descending order from left to right. Only survival from the above eight treatments with high triploidy rates was shown. Means sharing a same horizontal bar did not differ significantly (P > 0.05).

Table 5.   Three-way ANOVA table for triploidy (after square-root transformation) in day 3
                   H. rubra larvae in experiment 2.

Sources	Sum of squares	DF	Mean squares	F-*ratios*	P-*values*
DS	164.403	1	164.403	2,419.21	0.000
ST	32.54	4	8.135	119.71	0.000
TD	122.822	2	61.411	903.67	0.000
DS × ST	7.726	4	1.932	28.42	0.000
DS × TD	8.805	2	4.402	64.78	0.000
ST × TD	34.717	8	4.340	63.86	0.000
DS × ST × TD	11.291	8	1.411	20.77	0.000
Residual	4.077	60	0.068		

DS: CB dosage; ST: starting time; TD: treatment duration.

Table 6.   Three-way ANOVA table for survival in day 3 *H. rubra* larvae in experiment 2.

Sources	Sum of squares	DF	Mean squares	F-ratios	P-values
DS	754.003	1	754.003	15.14	0.0003
ST	1,114.52	4	278.631	5.59	0.0007
TD	307.753	2	153.876	3.09	0.0529
DS × ST	3,858.44	4	946.609	19.35	0.0000
DS × TD	210.314	2	105.157	2.11	0.1300
ST × TD	1,166.93	8	145.866	2.93	0.0079
DS × ST × TD	974.352	8	121.794	2.44	0.0232
Residual	2,988.85	60	49.8124		

ST: starting time; DS: CB dosage; TD: treatment duration.

Table 7.   Summary of optimal conditions for triploidy induction in *H. rubra* using CB, with rates of triploidy and survival in day 3 larvae (means ± SD, *n* = 3). All the treatments were targeted at inhibition of PB2 formation.

Treatments	DS (mg L^{-1})	ST (min post-fertilization)	TD (min)	Triploidy (%)	Survival [1] (%)	Relative survival [2] (%)
Experiment 1						
15	0.5	15	40	89.5 ± 4.3[a]	2.7 ± 1.5[a]	20.1
17		25	20	85.3 ± 4.7[a]	6.9 ± 3.8[a]	52.1
18			40	84.8 ± 1.9[a]	3.5 ± 2.6[a]	26.5
Experiment 2						
21	0.5	15	40	96.5 ± 0.5[a]	22.6 ± 9.1[abc]	55.6
24		20	40	92.2 ± 5.7[abc]	18.5 ± 6.5[bc]	45.3
26		25	20	94.6 ± 0.6[ab]	30.2 ± 4.6[a]	74.1
27			40	87.1 ± 5.6[bc]	13.5 ± 3.1[c]	33.0
29		30	20	86.5 ± 2.9[c]	26.0 ± 4.7[ab]	64.0
30			40	88.8 ± 1.8[abc]	17.7 ± 4.3[c]	43.4

DS: CB dosage. ST: starting time. TD: treatment duration. Within a column in each experiment, means sharing a same superscript did not differ significantly (*P* > 0.05).
[1] Survival = yields of normal day 3 larvae from eggs.
[2] Relative survival = (survival of treatments/the diploid control) × 100. Survival of the diploid control in each experiment refers to Tables 1 and 4, respectively.
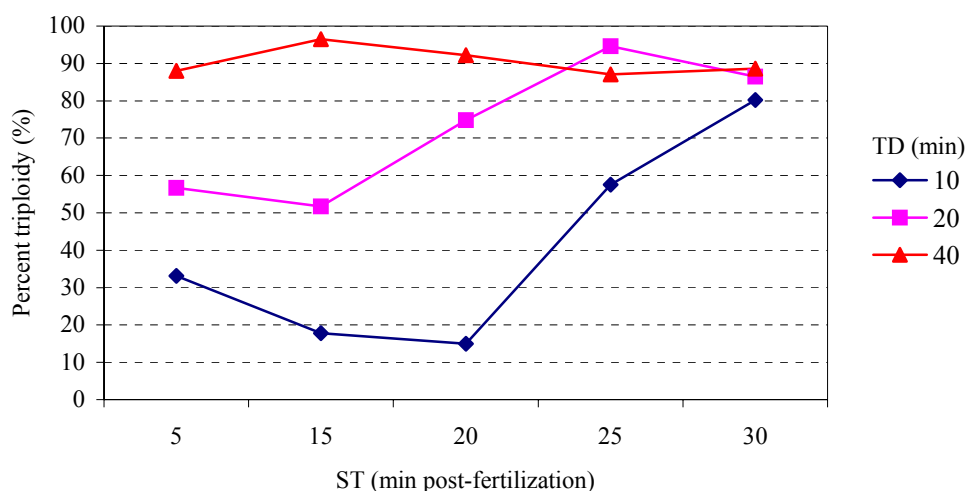
Figure 1. Interaction plot of starting time (ST) and treatment duration (TD) at 0.5 mg CB L^{-1} on triploidy. A: treatments targeted at inhibition of PB1 formation. B: treatment targeted at inhibition of both PB1 and PB2 formation. All the other treatment combinations were targeted at inhibition of PB2 formation.
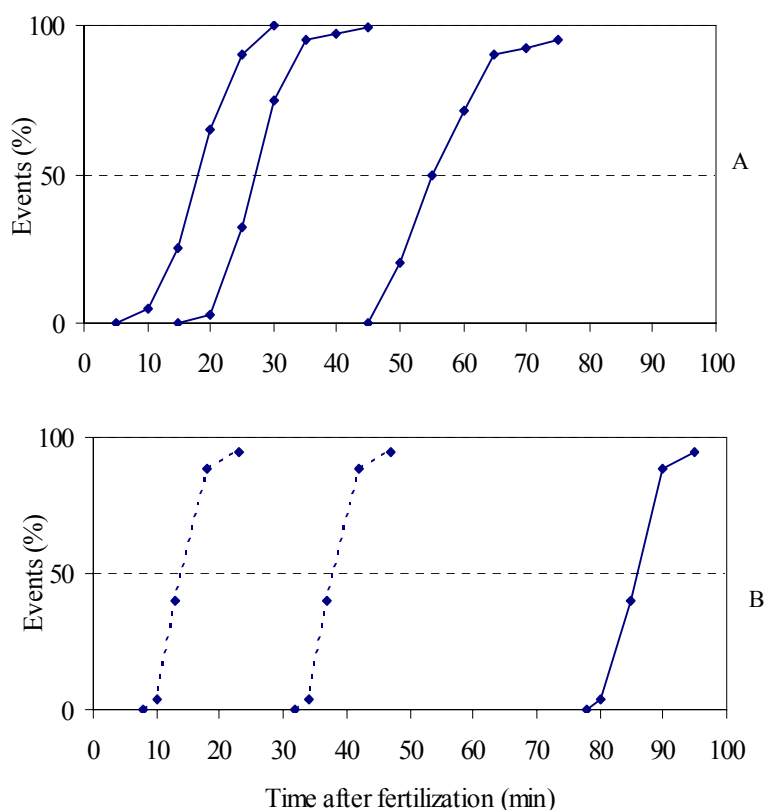
Figure 2 Kinetics of PB1 and PB2 extrusion, and first cleavage of (A) Pacific oyster *C. gigas* at 25°C (modified from Gérard *et al.* 1994); and (B) blacklip abalone *H. rubra* at 18°C. Left line: PB1; middle line: PB2; right line: first cleavage. The kinetic curve of first cleavage of *H. rubra* was based on a different batch of fertilized eggs from the present study, fixed in seawater containing 4% formalin at 5-min intervals from the onset of first cleavage (unpublished data). Dash lines were hypothesized.

4.1.2. Optimisation of triploidy induction in the blacklip abalone, *Haliotis rubra* (Leach), using 6-dimethylaminopurine (6-DMAP)

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4.1.2.1. Abstract

Optimal conditions for the use of 6-dimethylaminopurine (6-DMAP) in triploidy induction in the blacklip abalone *Haliotis rubra* (Leach, 1814), targeting inhibition of second polar body (PB2) formation were investigated. Two experiments were conducted at $17.5 - 18.5^{\circ}C$. In the first experiment treatments comprised factorial combinations of: (1) four dosages (DS) of 100, 150, 200 and 250 μM 6-DMAP; four starting times (ST) of 15, 20, 25 and 30 min post-fertilization; and two treatment durations (TD) of 20 and 30 min. In the second experiment treatments comprised factorial combinations of three DS's of 50, 100 and 150 μM 6-DMAP, three ST's of 15, 20 and 25 min post-fertilization, and three TD's of 10, 20 and 30 min. Day 3 larvae were sampled for ploidy status and survival. Percent triploidy was verified by flow cytometry. Results show that optimal induction protocols that yield both high rates of triploidy in combination with acceptable rates of survival were those commenced 15 or 20 min post-fertilization and continued for 20 or 30 min, using 100 μM 6-DMAP. These conditions induced rates of triploidy and relative survival of 80.5 – 93.3% and 36.5 – 40.2%, respectively, in the first experiment, and corresponding rates of 79.1 – 93.6% and 20.7 – 43.0%, respectively, in the second experiment. High percent triploidy was also obtained in a number of treatments using 150 μM 6-DMAP, but with overall survival rates generally were lower than treatments that used 100 μM 6-DMAP.

4.1.2.2. Introduction

The blacklip abalone, *Haliotis rubra* (Leach, 1814), fishery in southern Australia accounts for about one third of global wild fisheries catches. Aquaculture of *H. rubra* began in the early 1980's, and has expanded rapidly in recent years throughout southern Australia. As *H. rubra* is slow grower farmed stocks requiring 3 – 4 years to attain a preferred market size of 80 mm shell length and 80g live-weight (Fleming 2000). Therefore, techniques that can be used to enhance its growth rate are essential to improving production efficiency and long term economic sustainability of farming.

The cells of triploid plants and animals possess three sets of chromosomes in instead of the usual two (diploids). Triploidy is a potentially valuable asset for farming animals as it commonly imparts sterility or reduced sexual activity. In turn this means that nutrients, otherwise used for gonadal development, are channelled into somatic growth. Theoretically then adult triploid molluscs should grow faster than their diploid counterparts (Beaumont and Fairbrother 1991). Exposure of recently fertilised eggs to the cytotoxic chemical 6-dimethylaminopurine (6-DMAP) has been shown to be effective in inducing triploidy both in bivalves (Desrosiers *et al.* 1993) and gastropods including several species of abalone (Zhang *et al.* 1998; Yan and Chen 2002; Norris and Preston 2003). The aim of this study was to optimise the use of 6-DMAP to induce triploidy in the blacklip abalone *H. rubra*.

4.1.2.3.   *Materials and methods*

Broodstock, gamete production and fertilization

Two experiments were conducted on separate occasions. First generation (F1) hatchery reared *H. rubra,* conditioned in a recirculating seawater system at 15 – 17°C (Savva *et al.* 2000) were used as broodstock. The hydrogen peroxide method of Morse *et al.* (1977), with further modification by Tong *et al.* (1992), was applied to induce spawning.

In both experiments, eggs collected from four females were pooled in a beaker, adjusted to a density of about 500 eggs L^{-1}, and then fertilized by adding sperm mixed from several males at final concentrations of approximately 1 or 3 × 10^5 spermatozoa L^{-1}. Five minutes after sperm addition, aliquots comprising 10-mL of egg suspension (about 5,000 activated eggs) were dispensed into 140-mL 'mini' down-wellers fitted with 98-μm mesh screens. The down-wellers were kept in clean seawater before triploidy induction treatment was commenced. Seawater was filtered to 1 μm (nominal) via a cartridge filter and treated with UV light generated by a 40-W UV lamp. Temperature was kept at 17.5 – 18.5°C during spawning, fertilization and treatment.

Triploidy induction and larval rearing

It was previously observed (see Section 4.1.1) that *H. rubra* eggs begin to extrude the first and second polar bodies (PB1and PB2) about 7 – 9 and 28 – 32 min, respectively, after contact with sperm, and that first cleavage occurs 75 – 85 min post-fertilization, under a standard hatchery temperature regime of 17 – 19°C. Therefore, factorial combinations of four dosages (DS) of 100, 150, 200 and 250 μM DMAP, four starting times (ST) of 15, 20, 25 and 30 min post-fertilization, and two treatment durations (TD) of 20 and 30 min, were applied in the first experiment to inhibit PB2 extrusion. This generated a total combination of 32 treatments, to which an untreated diploid control was added. This array of treatments did not however provide information on the lower effective threshold concentration for the use of 6-DMAP nor equivalent information on treatment duration. A second experiment was therefore devised to address these deficiencies with treatments comprising factorial combinations of three DS's of 50, 100 and 150 μM 6-DMAP, three ST's of 15, 20 and 25 min post-fertilization, and three TDs of 10, 20 and 30 min, a total of 27 treatments, plus a diploid control.

In both experiments, three replicates mini down-wellers were applied to each treatment and the diploid control. Groups of three down-wellers were strung onto a plastic rod for easy handling and to facilitate accurate control over timing. At each ST, down-wellers were immersed in 1-L seawater baths containing appropriate concentrations of 6-DMAP (Sigma Aldrich, Australia). The down-wellers were washed in clean seawater after treatment, and then installed into a flow-through seawater system and provided with an average flow rate of 30 – 80 mL min^{-1} per down-weller, at 16.7 – 17.5°C through the period of incubation and a further two days of larval rearing. Water levels of the system were adjusted so that seawater in each down-weller was maintained at a volume of about 100 mL.

Sampling

Samples were taken approximately 70 h post-fertilization (day 3 larvae) to assess triploidy by flow cytometry (FCM), and survival (see Section 4.1.1 for detailed methods).

Statistical analysis

A fixed three-way ANOVA model was used to analyse the effects of the three induction factors (DS, ST and TD) and their interactions on rates of triploidy induction and survival, using the statistical package *Statgraphics Plus for Windows* 4.1 (Manugistics Inc., USA), followed by a

Fisher's least significant difference (LSD) multiple range test to compare differences between means with significance set at $P < 0.05$. Data were square-root transformed if necessary to remove heterogeneity of variances as confirmed by Bartlett's test. The diploid control introduced in each experiment was a common control, serving to locate the diploid peaks for flow cytometry analysis and to indicate the egg quality by presenting survival data, rather than for statistical comparison against the treatments.

4.1.2.4. *Results*

Early cytology

PB1 and PB2 extrusions were first observed 9 and 31 min post-fertilization, respectively, in the first experiment, and 8 and 32 min, respectively, in the second experiment.

Experiment 1

Triploidy induction

Percent triploidy in normal day 3 larvae ranged from 29.6 to 93.3% over the 32 treatments (Table 1; Figure 1). No detectable triploid peaks could be observed in the diploid control.

Since the use of 200 and 250 µM 6-DMAP (treatments 17 – 32, Table 1) led to unacceptably low larval survival (< 1%) on most occasions, triploidy and survival from all the associated 16 treatments were eliminated from the statistical analysis. Three-way ANOVA on the remaining data showed that DS, ST, and interactions of DS and ST and of DS, ST and TD, had highly significant effects ($P = 0.0007$) on percent triploidy. The effects of TD, and interactions between DS and TD and between ST and TD were not significant ($P > 0.05$) (Table 2).

Use of 100 µM 6-DMAP induced high triploidy rates of 80.5 – 93.3% in treatments commenced 15 or 20 min post-fertilization and continued for 20 or 30 min (treatments 1 – 4, Table 1). Use of 150 µM 6-DMAP in treatments started 15 or 20 min post-fertilization and continued for 20 or 30 min (treatments 9 – 12, Table 1), and in a treatment initiated 25 min post-fertilization and applied for 30 min (treatment 14, Table 1), also showed high triploidy rates of 75.7 – 89.3%. Although triploidy rates ≥ 80.4% were not uniform, they were all significantly greater ($P < 0.05$) than in all other treatments where triploidy rates were < 75.7% (Table 1).

These results showed that 100 and 150 µM 6-DMAP were within the suitable range, and that 200 and 250 µM 6-DMAP were too high. Accordingly these two DS's were omitted and an additional lower DS of 50 µM 6-DMAP was included in the second experiment. As a ST of 30 min post-fertilization appeared to be too late to start treatment it was also omitted from this second experiment. Nevertheless, yields of triploidy were not very sensitive to the two treatment durations of 20 and 30 min chosen; hence a shorter duration of 10 min was added in the second experiment.

Survival

Survival rates of day 3 normal larvae varied from 0 to 31.6% in the 32 treatments, and that of the diploid control was 64.2%. The corresponding range in relative survival rates was 0 to 49.2% (Table 1). After removing survival date from treatments using 200 and 250 µM 6-DMAP from the analysis, three-way ANOVA showed that there was a highly significant effect ($P \leq 0.0001$) of DS, and a significant effect ($P < 0.05$) of interaction between DS and ST on survival. However, effects of all the other factors, including interaction among DS, ST and TD, were not significant ($P > 0.05$) (Table 3).

Experiment 2

Triploidy induction

Yields of triploids in day 3 larvae ranged from 21.6 to 96.0% across the 27 treatments (Table 4). As in the first experiment, no triploid peaks were detected in the diploid control. Three-way ANOVA revealed that percent triploidy (after square-root transformation) was highly affected ($P \leq 0.0001$) by DS, ST and TD, and by all their interactions (Table 5).

Fifty μM 6-DMAP was not sufficiently effective for triploidy induction in *H. rubra*, since all the nine treatments using this concentration (treatments 1 – 9, Table 4) produced low triploidy rates $\leq 30.3\%$. Consistent with results of the first experiment, a dosage of 100 μM 6-DMAP induced high triploidy rates of 79.1 – 93.6% in treatments commenced 15 or 20 min post-fertilization and continued for 20 or 30 min (treatments 11, 12, 14 and 15, Table 4), but not in those with starting time of 25 min post-fertilization or with treatment duration of 10 min (treatments 10, 13, 16, 17 and 18, Table 4). All treatments using 150 μM 6-DMAP (treatments 19 – 27, Table 4) gave rise to high triploidy rates of 84.8 – 96.0%. Although these triploidy rates were not uniform, they were significantly higher ($P < 0.05$) than any of those < 79.1% (Table 4).

Survival

Survival rates of day 3 larvae ranged from 3.1 to 43.7% across the 27 treatments, while that of the diploid control was 50.8%. Relative survival rates ranged from 6.1 – 86.1% (Table 4).

A three-way ANOVA found highly significant effects ($P = 0.0031$) of DS, TD, and of the interaction of DS, ST and TD on survival. However, effects of all the other factors were not significant ($P > 0.05$) (Table 6).

Optimal induction conditions

Results of both the experiments confirmed that treatments started 15 or 20 min post-fertilization and continued for 20 and 30 min using 100 μM 6-DMAP, were optimal s for triploidy induction in *H. rubra* (Table 7). Use of these treatment conditions in combination resulted in rates of triploidy and relative survival rates of 80.5 – 93.3% and of 36.5 – 40.2%, respectively, in the first experiment, and of 79.1 – 93.6% and 20.7 – 43.0%, respectively, in the second experiment. In both experiments, significant differences ($P < 0.05$) could be found between these high rates of triploidy; however, the associated survival rates were uniform.

Although the use of 150 μM 6-DMAP could also induce high percent triploidy (> 75%) (Tables 1 and 4), the resultant overall survival rates (< 20% in terms of relative survival) were generally lower than those using 100 μM 6-DMAP. Therefore, care should be taken with these treatment conditions if larval survival is of concern. Nevertheless, one of these treatments (treatment commenced 25 min and applied for 20 min) did not bring about comparable yields of triploidy, namely, 69.1 (treatment 13, Table 1) and 96% (treatment 26, Table 4) in experiments 1 and 2, respectively.

4.1.2.5.   Discussion

Desrosiers *et al.* (1993) first demonstrated 6-DMAP to be an efficient and practical inducer of triploidy in the Pacific oyster *Crassostrea gigas*, blue mussel *Mytilus edulis* and giant scallop *Placopecten magellanicus*,. The authors recommended this puromycin analog as a preferred alternative to the more commonly used but highly toxic induction agent cytochalasin B (CB) for triploidy induction in bivalve molluscs, because of its comparable efficacy and because it is much safer to handle than CB. The efficacy of 6-DMAP in inducing triploidy has been confirmed in a

number of molluscan shellfish important to aquaculture, however, with triploidy induction rates varying greatly among species, from as low as 15% in the eastern oyster *Crassostrea virginica* and as high as 99% in the Pacific oyster *C. gigas* (Table 8).

In the present study, treatments that included a DS of 100 μM 6-DMAP were found best for *H. rubra* inducing triploidy rates of 79.1 – 93.6%. High percent triploidy was also obtained in a number of treatments using 150 μM 6-DMAP. These results showed that the efficiency of 6-DMAP for triploidy induction in *H. rubra* is high and comparable to rates of 84.8 – 96.5% previously achieved with CB (see Section 4.1.1). These results contrast with those achieved with other molluscs such as by Nell *et al.* 1996 who achieved triploidy induction rates of 40 – 73% in Sydney rock oyster *Saccostrea commercialis* (=*glomerata*) when using 6-DMAP compared to rates of 75 – 85% when using CB.

As illustrated by a summary of published data (Table 8) the optimal concentration of 100 μM 6-DMAP found here for *H. rubra* is lower than that of 200 – 600 μM reported for marine bivalves and of 200 – 300 μM reported for tropical abalone (*H. asinina* and *H. diversicolour aquatilis*), but close to 125 – 150 μM in the temperate abalone *H. discus hannai*. Higher concentrations of 200 and 250 μM 6-DMAP were shown to reduce larval survival to unacceptably low rates and are thus should be considered as toxic to *H. rubra*. Zhang *et al.* (1998) also found that no veligers of *H. discus hannai* could survive the use of 6-DMAP concentrations of 300 μM and beyond. These results seem to imply that temperate abalone are less tolerant to 6-DMAP than marine bivalves and their tropical counterparts.

Results of this study found that the interaction of the three induction factors (DS, ST and TD) had highly significant effects ($P \leq 0.0007$) on percent triploidy in day 3 *H. rubra* larvae associated with the use of 6-DMAP. However the practical significance of this is completely different from that of similar levels of interaction shown previously (see Section 4.1.1) with the use of CB. Successful triploidy induction treatment with CB that prevents extrusion of PB2 across large batches of *H. rubra* zygotes can be initiated between 15 – 30 min post-fertilization but must be extended to after about 45 min post-fertilization (ST + TD $\geq$ 45 min post-fertilization). By contrast successful induction of triploidy with 6-DMAP is limited to earlier starting times of 15 – 20 min post-fertilization for 100 μM or 15 – 25 min post-fertilization for 150 μM 6-DMAP (Tables 1 and 4). While 6-DMAP treatments ended 45 min post-fertilization provided no guarantee of high triploidy rates (for instance, treatments 17 and 18, Table 4); those discontinued before 45 min post-fertilization (ST + TD < 45 min post-fertilization), were still able to induce high percent triploidy (for example, treatments 11, 14, 19 and 20, Table 4). These results collectively suggest that the mechanisms by which 6-DMAP induces triploidy differ fundamentally from those of CB.

As to the starting time (ST), in the set of serial four treatments starting 15, 20, 25 and 30 min post-fertilization, respectively, but sharing a same treatment duration of 30 min, using 100 μM 6-DMAP (treatments 2, 4, 6 and 8, Table 1), it was found that triploidy rates actually showed a declining trend with lapsed time post-fertilization viz. 15 min (93.3%) > 20 min (89.4%) > 25 min (48.6%) > 30min (30.4%). Since all the treatments lasted long enough to cover the entire period of time for PB2 extrusion (ST + TD $\geq$ 45 min post-fertilization), it is unlikely that the eggs were still able to release their PB2 and escape as diploids after treatment ended. It appears more likely that 6-DMAP did not take action immediately (for whatever reason), with the lag time spanning quite a few minutes at this concentration.

A probable consequence, for treatments starting 25 and 30 min post-fertilization, is that large proportion of the eggs had already extruded their PB2 when 6-DMAP actually began to work (PB2 was released about 32 min post-fertilization in the present study) and survived as diploids. This hypothesis can account for the low rates of triploidy induced by these two treatments. On the other hand, the use of 150 μM 6-DMAP was able to induce high rates of triploidy in treatments initiated

not only 15 – 20 but also 25 min post-fertilization (Tables 1 and 4). This again highlights the critical linkage between starting time (ST) and 6-DMAP dosages (DS), with a trend of faster reaction time being imposed with higher concentrations of 6-DMAP. Hence, if only a single starting time is included in experiments to optimise use of 6-DMAP, and if this ST is chosen arbitrarily or improperly (too late), valuable information on the induction efficiency of 6-DMAP at lower concentrations will be missed.

This issue could in turn explain low triploidy induction rates of 56% obtained by Zhang *et al.* 1998 with the Pacific abalone *H. discus hannai* using lower concentrations of 125 – 150 μM 6-DMAP and of 15% for the eastern oyster *C. virginica* reported by Scarpa *et al.* 1995 using 200 μM 6-DMAP. A common response to such poor triploidy induction rates is to use higher concentrations of 6-DMAP. Unfortunately, for these two species that appear particularly sensitive to cytotoxic effects of 6-DMAP, the cost of increasing DS is unacceptably high mortality, as showed here in relation to *H. rubra*.

The ST adopted by Zhang *et al.* 1998 for *H. discus hannai* was the observed time of 50% extrusion of PB1, a criterion originally developed by Allen *et al.* 1989 to maximise triploid yield in the Pacific oyster *C. gigas* using CB. The low triploidy rates (56%) obtained in *H. discus hannai* by Zhang *et al.* 1998 when using this criterion illustrate its shortcomings being appropriate only if coupled to the use of CB and in application to bivalve molluscs.

A model proposed by Desrosiers *et al.* (1993) to explain triploidy induction by 6-DMAP is that when the eggs are treated at metaphase II, the extrusion of PB2 is inhibited and only one female pronucleus is formed. The model further postulates that the diploid female pronucleus fuses with the haploid male pronucleus to produce triploid zygotes. This model however appears to ignore the fact that 6-DMAP can disrupt the metaphase spindles (Néant *et al.* 1989; Szollosi *et al.* 1991), and therefore arrest the movement of the dyads (each has duplicated chromatids) during meiosis (e.g., blocking metaphase-anaphase transition), as observed by Cai and Beaumont (1996). By contrast CB does not interfere with spindle formation nor inhibit the separation of chromosomes during meiosis (Guo *et al.* 1992; Longo *et al.* 1993).

Triploids can be produced after the second set of chromatids are separated and their expulsion as PB2 is suppressed. In this case the second set of chromatids is retained to form a diploid rather than a haploid egg nucleus (Beaumont and Fairbrother 1991). Chemical induction of triploidy when applied to fertilized eggs during metaphase II and when sufficiently protracted for all eggs to have formed metaphase spindles in preparation for polar body formation (in the case of *H. rubra* those treatments where ST + TD < 45 min post-fertilization) (Longo *et al.* 1993; Désilets *et al.* 1995), three main types of eggs are produced. One type of egg suffers arrested development at metaphase II when spindles are disrupted too soon. The second type of eggs suffer complete separation of the second set of chromatids when reaching anaphase II. The third type of eggs are intermediate forms of the first two in which the second set of chromatids are not completely separated. The first and the last types of eggs are likely to be unviable, either ceasing to develop, or developing abnormally and eventually dieing during early larval development. Only the second type of eggs have a chance to survive, and only as triploids. This also renders high percent triploidy, regardless of how many triploids are produced. To maximize the production of triploids, the development of the eggs needs to be as synchronous as possible (Desrosiers *et al.* 1993).

For best results initiation of triploidy induction should also coincide with the time when a maximum proportion of eggs are undergoing chromosome segregation during meiosis. 6-DMAP used at higher concentrations acts too fast (disrupts metaphase spindles too quickly) resulting in fewer viable eggs that are able to complete metaphase-anaphase transition. This may explain the lower overall survival rates imposed by the use of 150 and 100 μM 6-DMAP in the present study (Tables 1 and 4). Worse still, even if numbers of diploids that survive treatment are low diploids

can still be predominant if only a small percentage of triploids is generated, as was the case for treatments that used 200 and 250 μM 6-DMAP in experiment 1 (Table 1). Further investigation of the timing of sequential meiotic events and of the orientation and movement of chromosomes following treatment will help unravel the precise mechanisms by which 6-DMAP induces triploidy.

4.1.2.6. *References*

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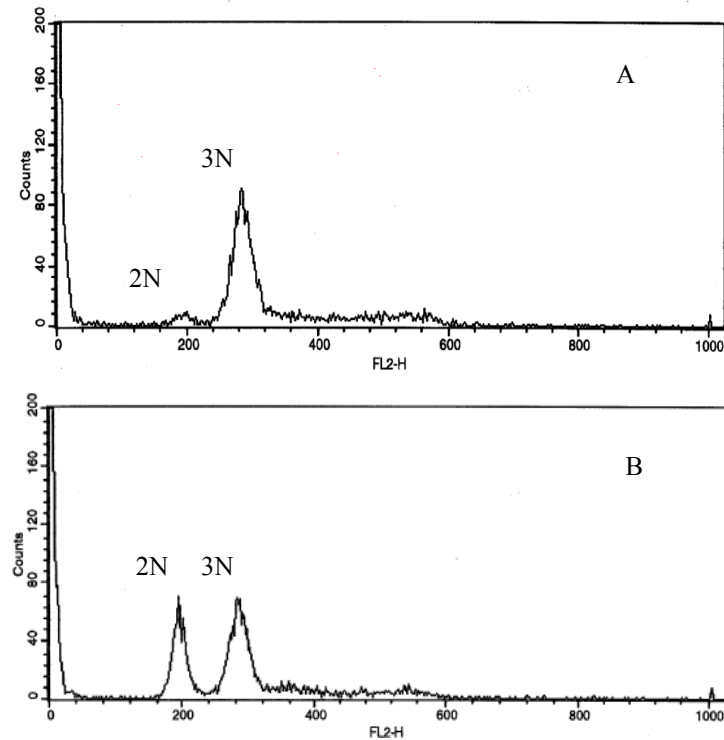
Figure 1.   Flow cytometric histograms of (pooled) day 3 *H. rubra* larvae with percent triploidy of (A) 93.8, and (B) 63.0%. Percent triploidy was determined as the proportion of triploid cells (falling within the range between 240 and 400 on the FL2-H axis that represents for the relative DNA contents) among total cells (falling within the range between 140 and 400 on the FL2-H axis) analysed. Cells falling outside the range of 140 – 400 on the FL2-H axis were considered debris and therefore uncounted. The peak positions of diploid (2N) were about 193 and triploid (3N) 282.

Table 1. Rates of triploidy and survival (means ± SD, *n* = 3) in day 3 *H. rubra* larvae at different treatment combinations of 6-DMAP dosage (DS), starting time (ST) and treatment duration (TD), and results of Fisher's LSD multiple range tests for triploidy and survival in experiment 1.

Treatments	DS (μM)	ST (min)	TD (min post-fertilization)	Triploidy[1] (%)	Survival[2] (%)	Relative survival[3] (%)
1		15	20	80.5 ± 8.1	25.8 ± 6.2	40.2
2			30	93.3 ± 3.3	23.4 ± 8.4	36.5
3		20	20	81.1 ± 2.2	24.5 ± 10.2	38.1
4	100		30	89.4 ± 10.9	24.2 ± 7.4	37.6
5		25	20	58.9 ± 1.7	15.6 ± 4.1	24.2
6			30	48.6 ± 1.5	17.9 ± 6.2	27.9
7		30	20	37.3 ± 2.1	24.8 ± 7.7	38.6
8			30	30.4 ± 2.3	31.6 ± 11.6	49.2
9		15	20	80.4 ± 7.4	4.3 ± 5.9	6.7
10			30	75.7 ± 9.6	2.5 ± 2.2	3.9
11		20	20	77.6 ± 12.2	3.6 ± 1.5	5.5
12			30	83.0 ± 2.1	6.7 ± 2.7	10.4
13	150	25	20	69.1 ± 5.7	11.4 ± 1.7	17.8
14			30	89.3 ± 3.5	11.3 ± 4.8	17.5
15		30	20	68.0 ± 2.3	10.2 ± 5.1	15.9
16			30	66.7 ± 9.7	9.8 ± 2.4	15.2
17		15	20	60.5	1.3 ± 0.4	2.1
18			30	55.6	0.3 ± 0.4	0.5
19		20	20	43.2 ± 5.8	11.7 ± 6.9	18.2
20	200		30	43.0	0.6 ± 0.3	0.9
21		25	20	42.1	0.6 ± 0.5	0.9
22			30	34.9	0.3 ± 0.5	0.5
23		30	20	50.4	0.2 ± 0.3	0.2
24			30	49.1	0.2 ± 0.3	0.2
25		15	20	64.7	0.6 ± 0.3	0.9
26			30	58.5	0.3 ± 0.3	0.5
27		20	20	46.4	0.5 ± 0.7	0.8
28	250		30	29.6	0.4 ± 0.2	0.5
29		25	20	40.4	0.4 ± 0.4	0.7
30			30	30.3	0.5 ± 0.0	0.7
31		30	20	37.9	0.2 ± 0.3	0.2
32			30	---	0	0
Diploid control				0	64.2 ± 4.4	100

Results of Fisher's LSD multiple range test for triploidy[4]

Treatments	2	4	14	12	3	1	9	11	10
Triploidy (%)	93.3	89.4	89.3	83.0	81.1	80.5	80.4	77.6	75.7

Results of Fisher's LSD multiple range test for survival[5]

Treatments	1	3	4	2	14	12	9	11	10
Survival (%)	25.8	24.5	24.2	23.4	11.3	6.7	4.3	3.6	2.5

[1] Means without SD: larvae from the three replicates were pooled due to poor survival.

[2] Survival = yields of normal day 3 larvae from eggs.

[3] Relative survival = (survival of treatments/the diploid control) × 100.

[4] Treatments 17 – 32 were not included in the analysis. Triploidy rates were arranged in a descending order from left to right. Means sharing a same horizontal bar did not differ significantly (*P* > 0.05). Triploidy rates below 75.7% were all significantly lower (*P* < 0.05) than those over 80.4% (but some were not significantly lower than 77.6 or 75.7%) and were not shown.

[5] Treatments 17 – 32 were not included in the analysis. Survival was arranged in a descending order from left to right. Only survival from the above nine treatments with high triploidy rates was shown. Means sharing a same horizontal bar did not differ significantly (*P* > 0.05).

Table 2.   Three-way ANOVA table of triploidy in day 3 *H. rubra* larvae in experiment 1.

Sources	Sum of squares	DF	Mean squares	F-*ratios*	P-*values*
DS	1,523.25	1	1,523.25	36.94	0.0000
ST	8,464.6	3	2,821.53	68.42	0.0000
TD	103.841	1	103.841	2.52	0.1224
DS × ST	4,098.74	3	1,366.25	33.13	0.0000
DS × TD	46.8075	1	46.8075	1.14	0.2947
ST × TD	210.308	3	70.1025	1.70	0.1867
DS × ST × TD	911.547	3	303.849	7.37	0.0007
Residual	1,319.63	32	41.2385		
Total	16,678.63	47			

DS: 6-DMAP dosage; ST: starting time; TD: treatment duration. Treatments using 200 and 250 μM 6-DMAP (treatments 17 – 32, Table 1) were not included in the analysis.

Table 3.   Three-way ANOVA table of survival in day 3 *H. rubra* larvae in experiment 1.

Sources	Sum of squares	DF	Mean squares	F-*ratios*	P-*values*
DS	3,065.6	1	3,065.6	78.66	0.0000
ST	214.344	3	71.4481	1.83	0.1610
TD	9.72	1	9.72	0.25	0.6209
DS × ST	462.265	3	154.088	3.95	0.0166
DS × TD	6.02083	1	6.02083	0.15	0.6969
ST × TD	43.2117	3	14.4039	0.37	0.7755
DS × ST × TD	47.3475	3	15.7825	0.40	0.7504
Residual	1,247.2	32	38.975		
Total	5,095.71	47			

DS: 6-DMAP dosage; ST: starting time; TD: treatment duration. Treatments using 200 and 250 μM 6-DMAP (treatments 17 – 32, Table 1) were not included in the analysis.

Table 4.   Rates of triploidy and survival (means ± SD, $n = 3$) in day 3 *H. rubra* larvae at different treatment combinations of 6-DMAP dosage (DS), starting time (ST) and treatment duration (TD), and results of Fisher's LSD multiple range tests for triploidy and survival in experiment 2.

Treatments	DS (μM)	ST (min)	TD (min post-fertilization)	Triploidy (%)	Survival [1] (%)	Relative survival [2] (%)
1		15	10	22.9 ± 1.7	31.1 ± 10.9	61.3
2			20	30.3 ± 2.2	21.4 ± 11.1	42.1
3			30	30.3 ± 0.8	29.9 ± 7.1	58.8
4		20	10	28.7 ± 0.8	41.6 ± 4.4	81.9
5	50		20	23.8 ± 1.0	29.5 ± 7.8	58.1
6			30	24.5 ± 1.1	30.6 ± 9.1	60.3
7		25	10	22.2 ± 1.3	40.9 ± 8.8	80.5
8			20	22.6 ± 0.2	43.7 ± 12.3	86.1
9			30	21.6 ± 1.4	10.1 ± 5.3	19.9
10		15	10	39.0 ± 0.3	26.9 ± 17.2	53.0
11			20	93.6 ± 0.9	21.9 ± 8.7	43.0
12			30	92.3 ± 1.3	15.3 ± 4.3	30.2
13		20	10	48.2 ± 1.8	14.1 ± 8.9	27.7
14	100		20	79.1 ± 5.6	16.9 ± 9.6	33.3
15			30	84.6 ± 2.8	10.5 ± 1.8	20.7
16		25	10	43.3 ± 0.9	14.1 ± 4.4	27.7
17			20	64.6 ± 1.7	11.9 ± 7.4	23.3
18			30	60.2 ± 4.6	11.4 ± 5.3	22.4
19		15	10	85.6 ± 2.7	14.8 ± 1.9	29.2
20			20	91.3 ± 5.1	7.8 ± 5.7	15.3
21			30	93.1 ± 2.2	4.3 ± 2.0	8.5
22		20	10	94.5 ± 4.1	15.1 ± 5.1	29.7
23	150		20	90.4 ± 3.3	3.1 ± 0.9	6.1
24			30	84.8 ± 1.3	4.1 ± 2.0	8.0
25		25	10	93.8 ± 2.7	8.6 ± 7.0	17.0
26			20	96.0 ± 1.3	6.7 ± 2.9	13.1
27			30	91.7 ± 4.2	8.8 ± 4.8	17.3
Diploid control				0	50.8 ± 5.5	100

Results of Fisher's LSD multiple range test for triploidy (after square-root transformation)[3]

Treatments	26	22	25	11	21	12	27	20	23	19	24	15	14
Triploidy (%)	96.0	94.5	93.8	93.6	93.1	92.3	91.7	91.3	90.4	85.6	84.8	84.6	79.1

Results of Fisher's LSD multiple range test for survival[4]

Treatments	11	14	12	22	19	15	27	25	20	26	21	24	23
Survival (%)	21.9	16.9	15.3	15.1	14.8	10.5	8.8	8.6	7.8	6.7	4.3	4.1	3.1

[1] Survival = yields of normal day 3 larvae from eggs.

[2] Relative survival = (survival of treatments/the diploid control) × 100.

[3] Triploidy rates were arranged in a descending order (in the original form) from left to right. Means sharing a same horizontal bar did not differ significantly ($P > 0.05$). Triploidy rates below 79.1% were significantly lower ($P < 0.05$) than any of those listed and were not shown.

[4] Survival was arranged in a descending order from left to right. Only survival from the above 13 treatments with high triploidy rates was shown. Means sharing a same horizontal bar did not differ significantly ($P > 0.05$).

Table 5.   Three-way ANOVA table of triploidy (after square-root transformation) in day 3 *H. rubra* larvae in experiment 2.

Sources	Sum of squares	DF	Mean squares	F-*ratios*	P-*values*
DS	290.463	2	145.231	5,836.81	0.0000
ST	3.1136	2	1.5568	62.57	0.0000
TD	10.9467	2	5.47336	219.97	0.0000
DS × ST	4.13841	4	1.0346	41.58	0.0000
DS × TD	19.6487	4	4.91217	197.42	0.0000
ST × TD	4.64624	4	1.16156	46.68	0.0000
DS × ST × TD	2.24315	8	0.280394	11.27	0.0000
Residual	1.34363	54	0.024882		
Total	336.543	80			

DS: 6-DMAP dosage; ST: starting time; TD: treatment duration.

Table 6.   Three-way ANOVA table of survival in day 3 *H. rubra* larvae in experiment 2.

Sources	Sum of squares	DF	Mean squares	F-*ratios*	P-*values*
DS	7,299.27	2	3,646.63	64.14	0.0000
ST	49.4156	2	24.7078	0.42	0.6498
TD	1,128.56	2	564.28	9.93	0.0002
DS × ST	566.946	4	141.586	2.49	0.0539
DS × TD	270.019	4	67.5048	1.19	0.3268
ST × TD	296.444	4	74.1111	1.30	0.2804
DS × ST × TD	1,546.58	8	193.329	3.40	0.0031
Residual	3,070.13	54	56.8543		
Total	14,220.13	80			

DS: 6-DMAP dosage; ST: starting time; TD: treatment duration.

Table 7.　　Summary of optimal conditions for triploidy induction in *H. rubra* using 6-DMAP, targeting at inhibition of PB2 formation, with rates of triploidy and survival in day 3 larvae (means ± SD, *n* = 3).

Treatments	DS (µM)	ST (min)	TD (min post-fertilization)	Triploidy (%)	Survival[1] (%)	Relative survival[2] (%)
Experiment 1						
1	100	15	20	80.5 ± 8.1[a]	25.8 ± 6.2[a]	40.2
2			30	93.3 ± 3.3[b]	23.4 ± 8.4[a]	36.5
3		20	20	81.1 ± 2.2[a]	24.5 ± 10.2[a]	38.1
4			30	89.4 ± 10.9[ab]	24.2 ± 7.4[a]	37.6
Experiment 2						
11	100	15	20	93.6 ± 0.9[a]	21.9 ± 8.7[a]	43.0
12			30	92.3 ± 1.3[a]	15.3 ± 4.3[a]	30.2
14		20	20	79.1 ± 5.6[b]	16.9 ± 9.6[a]	33.3
15			30	84.6 ± 2.8[b]	10.5 ± 1.8[a]	20.7

DS: 6-DMAP dosage; ST: starting time; TD: treatment duration. Within one column of each experiment, means sharing a same superscript did not differ significantly ($P > 0.05$).

[1] Survival = yields of normal day 3 larvae from eggs.

[2] Relative survival = (survival of treatments/the diploid control) × 100. Survival of the diploid control in each experiment refers to Tables 1 and 4, respectively.

Table 8.   A brief comparison of the effectiveness of 6-DMAP in inducing triploidy in molluscan shellfish important to aquaculture, targeting at inhibition of PB2 formation.

Species	Best results			Age at sampling	References
	DS (µM)	Triploidy (%)	Survival (%)		
ABALONE					
Haliotis asinina	200 – 250	89.1 – 96.4	96.5 – 97.1	Trochophores	Norris and Preston (2003)
H. discus hannai (Pacific abalone)	125 – 150	47.0 – 56.0	61.2 – 73.3	Trochophores	Zhang *et al.* (1998)
H. diversicolour aquatilis	300	90	85	Embryos	Yan and Chen (2002)
H. rubra (Blacklip abalone)	100	79.1 – 93.6	10.5 – 25.8	Day 3 larvae	The present study
CLAMS					
Panopea abrupta (Geoduck clam)	600	92.5	30.1	Day 3 larvae	Vadopalas and Davis (2004)
MUSSEL					
Mytilus edulis (Blue mussel)	600	--	---	---	Desrosiers *et al.* (1993)
	400	83.1	1.4	D-larvae	Braker *et al.* (2002)
OYSTERS					
Crassostrea gigas (Pacific oyster)	300	90	---	Day 1 larvae	Desrosiers *et al.* (1993)
	450	85	64	Embryos	Gérard *et al.* (1994)
	300 – 450	99	34	Day 1 larvae	Gérard *et al.* (1999)
	450	93.8	81	Trochophores	Tian *et al.* (1999)
C. virginica (Eastern oyster)	200	15	15	Day 2 larvae	Scarpa *et al.* (1995)
Saccostrea commercialis (Sydney rock oyster)	300 – 400	40 – 73	23 – 57	Day 5 larvae	Nell *et al.* (1996)
SCALLOPS					
Chlamys farreri	350	> 80	70	Embryos	Yang *et al.* (1999)
Placopecten magellanicus (Giant scallop)	400	95	---	Embryos	Desrosiers *et al.* (1993)

DS: 6-DMAP dosage.

4.1.3. Induction and evaluation of triploidy in the Australian blacklip abalone, *Haliotis rubra*: A preliminary study

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4.1.3.1. Abstract

Large batches of sibling triploid and diploid juvenile *Haliotis rubra* were produced to evaluate and compare their farming performance. Triploidy was induced by inhibiting extrusion of the second polar body (PB2) using either 150 μM 6-dimethylaminopurine (6-DMAP) or 0.5 mg/L cytochalasin B (CB). Best guess conditions were used in which treatments were started at 25 – 26 minutes after fertilization and continued for 20 minutes at $17.5 – 18.5^{o}C$. Triploid percentages, survival and growth were examined up to 22 weeks after settlement. Triploid percentages in day 2 larvae were 94 and 92% in 6-DMAP and CB treatments respectively. Overall survival from eggs to competent larvae was 30.0 and 55.5% for 6-DMAP and CB treatments respectively, compared to 64.8% for the diploids. Yields of 20 weeks post-settlement juveniles (from competent larvae) were less than 1.9% in the two triploid treatments and the diploid control. A shell length was less than 3.7 mm in all the three cases. At 22 weeks after settlement, triploid rates were 100% for 6-DMAP and 82.5% for CB treatments.

4.1.3.2. Introduction

In practice triploid bivalves generally exhibit increased growth as a result of retarded gonadal development rather than complete sterility as originally presumed (Beaumont and Fairbrother 1991). The degree of retardation varies with species. While triploid American oysters and Sydney rock oysters are functionally sterile (Barber and Mann 1991; Cox *et al.* 1996), triploid Pacific oysters on occasion realize high fecundity (Guo and Allen 1994a). This enables them to be used for producing tetraploids (Guo and Allen 1994b), which in turn provide a technique that ensures 100% triploid yields by crossing male tetraploids with female diploids (Guo *et al.* 1996; Nell 2002). The Pacific oyster industry throughout the world, especially in the United States, has already benefited greatly from triploid oysters (Nell 2002), in which the retarded gonadal development prolongs the annual period over which they are in marketable condition year-round (Allen and Downing 1986, 1990). In future, other bivalve farming industries are likely to take advantage of faster growth and/or improved meat quality of triploids (Barber and Mann 1991; Nell *et al.* 1994; Hand *et al.* 1998; Nell 2002).

In contrast to bivalves, information on the effects of triploidy on performance of farmed abalone and other gastropods is scant. Although triploidy induction has been attempted in Pacific abalone *Haliotis discus hannai* (Arai *et al.* 1986; Wang *et al.* 1990; Zhang *et al.* 1998), small abalone *H. diversicolor supertexta* (Yang *et al.* 1998), South African abalone *H. midae* (Stepto and Cook 1998), and Pacific red abalone *H. rufescens* (Maldonado *et al.* 2001), with thermal, CB or 6-DMAP treatment, few of the studies provided techniques that combined high triploid percentages with reasonable larval survival. Also lacking is an in depth evaluation of relative growth and reproduction performances of triploid and diploid sibling stock (Zhang *et al.* 1998). As a consequence, whether abalone farming industries can exploit the advantages conferred by triploidy, namely sterility or fast growth, has not yet been accurately assessed.

The blacklip abalone, *Haliotis rubra*, is an important fisheries species in Australia, currently accounting for 80% of the Australian abalone fisheries production and about 40% of wild fisheries production across the world. Its natural range extends from Coffs Harbour in New South Wales (NSW) around the south of Australia, including the coastal waters of Tasmania and the Bass Strait, and as far west as Rottnest Island off Perth in Western Australia (Shepherd 1973, 1975). In recent years, farming of two abalone species *H. rubra* and the greenlip abalone *H. laevigata* has become one of Australia's fastest growing aquaculture industries (Fleming 2000). However, like most other commercially important abalone species, the slow growth rate of *H. rubra* is a major obstacle to the profitable farming of this species.

At the outset of this experiment May 2002, large batches of triploid *H. rubra* and their diploid siblings were produced. The purpose was to evaluate and compare production performances of triploids and diploid siblings, thereby providing important information for assessing the potential of triploids for aquaculture production and for enhancement of depleted fisheries.

4.1.3.3.   Materials and methods

Broodstock, gamete production and fertilization

Wild collected *H. rubra* broodstock were conditioned in the recirculating seawater system of NSW Fisheries Abalone Research Unit (Shoal Bay, NSW, Australia), maintained at temperatures in the range $15 – 17^{o}C$ (Savva *et al.* 2000). A total of 16 females and seven males were induced to spawn by applying the hydrogen peroxide method of Morse *et al.* (1977), with further modification by Tong *et al.* (1992). Females were held individually, while males were held in pairs in 10 L tubs filled with 6 L of seawater, to which 40 mL of 2 M Tris was added to increase pH to 9.1. About 15 min after adding the Tris, 18 mL of freshly prepared 6% H_2O_2 was added into each tub. Three hours later, the tubs were completely rinsed and refilled with clean seawater. Spawning usually occurred after a further 0.5 – 2 hours.

A total of 7.4 million eggs collected from seven females were pooled and then divided into three buckets (about 2.5 million per bucket). Egg suspensions in each of the three buckets were made up to 5.0L with clean seawater. An 80 mL aliquot of fresh (< 30 min post-spawning) sperm pooled with equal contribution from four males at a concentration of 6×10^6 spermatozoa mL^{-1}, was added into each bucket to fertilize the eggs (day 0). This gave a final concentration of about 1×10^5 spermatozoa mL^{-1} and sperm/egg ratio of about 200:1. The eggs were washed 8 min post-fertilization. The three buckets of eggs were each used for 6-DMAP and CB induction of triploidy treatments, and the diploid control. Fertilization for CB treatment was staggered 15 min behind that for 6-DMAP treatment and the diploids to ensure accurate timing control during induction. Seawater was filtered to 1 μm (nominal) via cartridge filter and treated with UV light generated by a 40-W UV lamp, until larval rearing. Water temperature during fertilization and triploidy induction was $17.5 – 18.5^{o}C$.

Triploidy induction

In this experiment, 150 μM 6-DMAP (Zhang *et al.* 1998) and 0.5 mg/L CB (Stepto and Cook 1998) were used to inhibit PB2 formation for triploidy induction. From previous observations, *H. rubra* eggs generally were known to extrude PB1 and PB2 about 7 – 9 and 28 – 32 min, respectively, after contact with the sperm, under the standard hatchery temperature regime (17 – $19^{o}C$). Hence a starting time of 25 – 26 min post-fertilization was chosen to inhibit PB2 extrusion.

In the case of 6-DMAP treatment, the eggs were immersed in 4.0 L of seawater containing 150 μM 6-DMAP (Sigma Aldrich, Australia) for 20 min, started at 26 min post-fertilization. For CB treatment, the eggs were immersed in 2.0 L of seawater with 0.5 mg/L CB (Sigma Aldrich,

Australia) and 0.1% dimethylsulfoxide (DMSO) also for 20 min, but started at 25 min post-fertilization. The eggs were then washed in each of two changes of 5 L of seawater containing 0.1% DMSO for 20 min after treatment.

Rearing of larvae, post-larvae and juveniles

Fertilized eggs from each treatment were transferred into a 340 L cylindro-conical rearing tank filled with clean 1μm filtered and UV disinfected seawater. The diploids control was directly stocked into a same type of rearing tank after being washed. In all the three cases, developing embryos were incubated at $18.2 – 19.5^{o}$C, and provided with gentle slug-flow aeration. Water flow was resumed when trochophores were hatched out (19 – 20 hours post-fertilization). Once the larval shell had been completely formed (about 30 hours post-fertilization), the larvae were siphoned onto a 140 μm-mesh screen to remove debris particles and abnormal larvae. They were then rinsed with clean 1μm filtered and UV disinfected seawater and restocked into the same rearing tank that in the meantime had been washed, disinfected and rinsed clean of residual disinfectant with 1μm filtered and UV disinfected seawater. The rearing tank was provided with flowing water at $3 – 3.5$ L min^{-1} and a moderate level of aeration. Temperature in the rearing tanks was maintained at $17.3 – 18.5^{o}$C. At day 4, the rearing tanks were drained, cleaned, disinfected and rinsed as above once again. These larvae were again wet sieved and rinsed with 1μm filtered and UV disinfected seawater before restocking into the rearing tanks. The larvae were reared under the above conditions until competent for settlement on day 7 after spawning. Triploid percentage was examined in day 2 larvae and survival at a number of larval development stages.

Competent larvae from the three cases were each transferred into outdoor rectangular settlement tanks. Clear PVC plates ($600 \times 300 \times 2$ mm) were placed in advance into the tanks to develop a light film of adventitious benthic diatoms, serving as both settlement substrates and food for post-larvae. Since there was uncertainty over the likely settlement success of triploids, all available competent larvae from the two triploid treatments (Table 1) were used for settlement. All 0.74 million larvae from 6-DMAP treatment were stocked into a settlement tank containing 136 settlement plates (on eight racks), while 1.12 of 1.37 million larvae from the CB treatment were stocked into a second settlement tank of the same size and with the same number of PVC plates as 6-DMAP treatment. The balance 0.25 million larvae from CB treatment were used for other studies. The usual rates for stocking competent diploid larvae into such tanks for settlement was 0.25 million.

Due to limited availability of facilities, a third settlement tank which was half the size of the former two was used for diploid larvae, 0.25 million of which were stocked as a control batch. The settlement tanks were shaded with woven green 60% shade-cloth after introducing the larvae. No water flow was provided to the settlement tanks for the first two days after adding the larvae. Over this time, temperature ranged from $17.9 – 20.4^{o}$C. By the time water flow was resumed, few larvae remained in the water column. Ambient flow-through seawater was pre-filtered through a rapid-sand and a 10 μm (nominal) cartridge filter. Seawater temperature ranged from 20.4 to 22.4^{o}C in the first few days. Gentle to moderate fine aeration was applied continuously.

At 20 weeks post-settlement, juveniles were removed from PVC plates after being anesthetized with benthocaine (Heasman *et al.* 2004), and then transferred into three shallow raceways (2×0.5 m in base area and 0.5 m deep), each for 6-DMAP and CB treatments, and the diploids, where they were weaned and cultured with a commercial powder diet (Adam and Amos Abalone Foods, Southern Australian, Australia). Each raceway was equipped with 12 concrete bricks ($22 \times 11 \times 3$ cm) with simple legs that provided a 2 cm high cavity beneath. These served daylight shelters for the juveniles, that were maintained at a water depth of about 20 cm. Survival through the 20 weeks following settlement was determined by counting juveniles on 40 PVC plates randomly sampled from the settlement tanks for 6-DMAP and CB treatments, or 14 plates from the settlement tank

containing the diploids. In all the three cases, 15 juveniles were chosen at random from each of 4 randomly selected plates to determine initial mean shell height at weaning.

Triploid percentage was again assessed 22 weeks post-settlement (two weeks after the juveniles had been transferred into the raceways). The flow cytometry method used (see *Ploidy determination* methods below) enabled the ploidy status of individuals to be determined. Accordingly, the size of triploids and diploids within the CB treatment, could be distinguished. However, because triploid percentage of juveniles in 6-DMAP treatment was 100%, intra-treatment comparison of the relative growth of triploid and diploid juveniles was precluded.

Estimation of yields of post-larvae during early settlement

Yields of early post-larvae (newly metamorphosed larvae) were estimated using a number of replicate '140 mL mini' down-weller rearing devices. Fifty larvae were added to each down-weller. A $76 \times 26 \times 2$ mm glass microscope slide that had been allowed to develop bio-films dominated by adventitious benthic diatoms was inserted into each down-weller, to provide a settlement substrate and food for the larvae. This stocking rate was equivalent to 4,500 larvae per standard commercial nursery plate ($600 \times 300 \times 2$ mm), which was in turn within the optimum range previously determined (Heasman *et al.* 2004) for maximizing yields of ready to wean $1 - 3$ mm shell length *H. rubra* post-larvae. Each down-weller was provided with seawater flow at a rate of about 0.5 L min^{-1} from the second day after stocking the larvae. As previously stated, seawater used for this experiment was filtered to 1 µm and irradiated with UV light. Seawater temperature varied over the narrow range of 16 to 17.5°C. On each of days 2, 4, 8, and 16 after stocking, three replicate down-wellers from each of the two triploid treatments as well as from the diploid control were sampled to determine mean survival rates of post-larvae.

Ploidy determination

Triploidy status was determined by flow cytometry (FCM) (Allen 1983; Allen and Bushek 1992). Day 2 larvae that had just completed 180° torsion of the foot mass, were fixed in Carnoy's solution (3: 1 ratio of absolute methanol and acetic acid), in preparation for FCM using techniques of Yang *et al.* (2000). Briefly, about 1000 fixed larvae per sample were concentrated into a 1.75mL microtube, to which 1.0mL of 50% acetic acid was added. The tubes were then shaken vigorously to dislodge and dissociate cells. Cell suspensions were then centrifuged, and cell pellets re-suspended (washed) in marine phosphate buffered saline (MPBS) (Allen 1983). After re-centrifuging, cell pellets were stained with 0.75 of propidium iodide (PI) staining solution (2 parts CTX-100 stock: 1 part PI stock: 1 part RNAse stock with 10% of total volume DMSO, where CTX-100 stock = 0.1% sodium citrate and 0.1% Triton X-100 (Sigma) in distilled water, PI stock = 1 mg PI/CTX-100, RNAse stock = 1 mg RNAse (Sigma, type 1AS) per distilled water) (Allen 1994), re-suspended by mixing, and kept cold and dark before analyzing. Two samples were used for each treatment.

A variation on the above shaking method used to prepare abalone larvae for FCM was applied to the juveniles. Instead of the more usual practice of crushing or deep freezing the samples, whole live juveniles were individually inserted into 1.75 mL micro-tubes. A $0.6 - 0.8$ mL aliquot of PI staining solution (depending on the size of a juvenile) was added into each micro-tube. Several micro-tubes were then placed into a larger specimen bottle. By vigorous manual shaking of the specimen bottle using standardised speed and pattern of oscillation, the micro-tubes were bounced rapidly between the lid and floor of the bottle. This resulted in sufficient numbers of somatic cells being dislodged and dissociated into the PI staining solution to successfully apply FCM. Samples were then kept cold and dark, and filtered through a 35 µm-mesh to remove extraneous debris immediately before being analysed by FCM. Between 40 and 50 juveniles were used from each treatment. As shells of most juveniles remained intact after being shaken, they were stored frozen and measured later.

Statistical analysis

One-way ANOVA was used to test differences among post-larval yields of 6-DMAP and CB treatments, and the diploid control, on each sampling occasion. T-tests were used to compare size of triploids and diploids within the CB treatment, with significant difference set at *P* ≤ 0.05 level, using the statistical package *Statgraphics Plus for Windows 4.1* (Manugistics Inc, USA).

4.1.3.4.   Results

Triploid percentages of the larvae

Triploid rates in day 2 larvae were 94 and 92% for 6-DMAP and CB treatments respectively, (average of the two replicate samples of each treatment). These were quite consistent with the observation that the great majority of eggs (on which polar bodies could be clearly observed) in the two treatments had a single polar body at the two-cell stage. Eggs in the untreated diploids began to extrude PB1 and PB2 from 8 and 30 min post-fertilization, respectively.

Survival of the larvae

Larval survival data for the two triploid treatments and the diploid control are presented in Table 1. Hatch rates (trochophore yields) were slightly lower in 6-DMAP and CB treatments (70 and 77.5% respectively) than that in the diploid control (83.4%). However, the proportion of abnormal trochophores in the two triploid treatments was much higher (24.3 and 15.7% for 6-DMAP and CB treatments respectively) than for diploids (2.9%). Survival from trochophores to day 2 larvae was relatively low in the 6-DMAP treatment (54.1%), but of a similar high level in the CB treated and diploid control larvae (92.0 and 91.2% respectively). Survival from day 2 to competent larvae (day 7) was 79.2% and 77.8% in 6-DMAP and CB treatments respectively, The later were similar to the survival rate of 85.2% obtained with the diploids. At the end of larval rearing, 0.74, 1.37 and 1.60 million competent larvae were harvested from 6-DMAP and CB treatments and the diploid control respectively. These corresponded to overall rates of survival from eggs of 30.0, 55.5, and 64.8% respectively (Table 1).

Yields of post-larvae during early settlement

Yields of post-larvae all peaked 4 days post-settlement, with averages of 36.0, 39.3 and 49.3% for the 6-DMAP and CB treated larvae and for the diploid controls respectively. Although post-larval yields decreased only slightly from day 4 to 8 post-settlement, they subsequently decreased drastically from day 8 to 16 after settlement (many empty post-larval shell were observed in the samples). By this time yields had fallen to only 2.0, 7.3 and 4.6% for the 6-DMAP and CB treatments, and diploid controls respectively (Figure 1).

One-way ANOVA showed that there were no significant differences (*P* > 0.05) among post-larval yields of 6-DMAP and CB treatments, and the diploids, on each sampling occasion post-settlement.

Survival and size of the juveniles

Yields 20 weeks after settlement were 12,800 juveniles from 6-DMAP treatment, 13,100 from CB treatment, and 4,700 from the diploids. These represented survival rates from competent larvae of 1.7, 1.2, and 1.9 % respectively (Table 2).

Juveniles from the two triploid treatments were similar in size, averaging 3.6 – 3.7 mm in shell length and 2.7 mm in shell width, and were both considerably larger than diploids that had an average shell length of 2.3 mm and width of 1.8 mm (Table 2).

Triploid yields and sizes of triploids and diploids within CB treatment

When assessed 22 weeks after settlement, triploid percentages were 100% in 6-DMAP treatment, and 82.5% in CB treatment (Table 3; Figure 2).

When juveniles in the CB treatment were grouped according to individual ploidy status, it was found that diploids were larger than triploids, being about 17% greater in mean shell length, and 19% in shell width. However, the differences were not statistically significant ($P = 0.11$) (Table 4).

4.1.3.5.   Discussion

Triploidy induction

Until recently, there was no published information on triploidy induction in *H. rubra*. Triploidy induction protocols applied in the present study were best guesses based on available information on bivalves and other abalone species. Triploid percentages in day 2 larvae were more than 90% in both 6-DMAP and CB treatments. The results are better than those obtained with *H. discus hannai* by Zhang *et al.* (1998) using 6-DMAP (150 μM), and in *H. midae* by Stepto and Cook (1998) using CB (0.5 mg L^{-1}), with percent triploidy of 56% and 70.9% respectively. These results show that both 6-DMAP and CB are highly efficacious for inducing triploidy in *H. rubra*.

Under a standard hatchery temperature regime of 17 – 19°C, *H. rubra* eggs generally begin to extrude PB1 and PB2 7 – 9 and 28 – 32 minutes respectively, after the introduction of sperm (unpublished observations). Results of this experiment were consistent with this pattern with PB2 first observed about 30 minutes post-fertilization. The decision to initiate triploidy induction treatments 25 – 26 minutes post-fertilization and to continue them for a period of 20 minutes, proved appropriate in light of the high triploid percentages achieved.

Under the protocols applied, use of 6-DMAP resulted in lower overall yields of competent larvae (30.0%) than CB (55.5%) or control diploids (64.8%). The lower larval yields associated with use of 6-DMAP appeared to be a consequence of elevated mortality before day 2, that is, before the larvae had completed 180° torsion of the foot mass. Beyond this stage, survival in the two triploid treatments as well as the diploids was uniform (79.2 – 85.2%, Table 1). These results indicate that day 2 larvae can be used as an early landmark for evaluating the relative success of alternative triploidy induction protocols in *H. rubra*.

When reassessed at 22 weeks after settlement, triploid percentages of juveniles were 100% in the 6-DMAP treatment, and 82.5% in the CB treatment (Table 2). Changes of triploid percentages over time have also been reported in other studies. This is probably due to differential mortality of triploids and diploids, that is, either higher mortality in triploids than in diploids (Allen and Downing 1986), or higher mortality in diploids than in triploids (Nell *et al.* 1994; Cox *et al.* 1996). On the other hand, observed differences in triploid percent tage between day 2 larvae and post-larvae within treatments in the present study may also be attributable to different techniques used to assess ploidy status in larvae and juveniles; that is, FCM was performed on pooled larvae not on individual juveniles. As pointed out by Hand *et al.* (1999) percent triploidy may vary by 5% or more from the actual ploidy level when animals (larvae) are analyzed in groups, possibly due to the presence of aneuploids that may remain during early larval stages, FCM machine settings, and/or varying cell numbers that one individual contributes to the samples analyzed by FCM.

Although juvenile yields from the two triploid treatments were low, so was the yield of diploid control (1.9%). The underlying cause of these low yields was traced to high levels of mortality in all the three cases between 8 and 16 days after settlement (Figure 1). This critical period for the post-larvae spans change from endogenous feeding to exogenous feeding. These uniform results

suggest that extraneous factors, such as original larval (egg) quality, or inadequate preparation of the settlement plates, were responsible for the low juvenile yields at 20 weeks after settlement. Equivalent observations were made by the authors during the course of plate seeding experiments (Heasman *et al.* 2004), a rapid decline in survival from around 70 to 20% occurring during the second week after settlement.

Growth

Average shell length of the juveniles from both triploid treatments and control diploids 20 weeks after settlement (Table 2) were 3.6 – 3.7 and 2.7 mm in shell length respectively. These equate to growth rates of about 24 and 17 μm/day in the two triploid treatments and the diploid control respectively. These growth rates fall within the generalized range of approximately 15 – 30 μm/day reported for post-larval abalone fed on poor diatom films, but are much lower than those of 40 – 60 μm/day for post-larvae that graze on highly digestible diatom films (Kawamura *et al.* 1998). This indicates that the quality of biofilm food (naturally occurred benthic diatoms) provided in the present study might not have been adequate. On the other hand, as the 20-week period of nursery culture spanned the period of early winter to early spring, the relatively lower growth rates may also have stemmed from low seawater temperatures known to suppress growth in early post-larval *H. rubra* (Heasman *et al.* 2004). Despite these shortcomings, post-larvae from both triploid treatments still showed a trend of faster growth than the diploids at early post-rearing stage. Similar results were reported by Zhang *et al.* (1998) for Pacific abalone *H. discus hannai*. However, it remains to be seen how *H. rubra* from the two triploid treatments, and triploids within the CB treatment batch (Table 4), will perform, in the longer term or most importantly, average time to market size.

4.1.3.6.   References

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Table 1.   Larval survival of *H. rubra* after 6-DMAP and CB treatment.

Treatments	Trochophores		Day 2 larvae		Competent larvae (Day 7)		
	Hatch rates (%)	Abnormal rates (%)	Survival (%)	Relative survival (%)	Survival (%)	Relative survival (%)	Number produced (million)
6-DMAP	70.0	24.3	37.9	54.1	30.0	79.2	0.74
CB	77.5	15.7	71.3	92.0	55.5	77.8	1.37
Diploids	83.4	2.9	76.1	91.2	64.8	85.2	1.60

Survival: from eggs.   Relative survival: each from previous development stage.

Table 2.   Survival and average size (± SD) of juvenile *H. rubra* 20 weeks after settlement in 6-DMAP and CB treatments and the diploids.

Treatments	Number of competent larvae stocked (million)	Number of juveniles produced	Survival (%)	SL (mm) (*n* = 60)	SW (mm) (*n* = 60)
6-DMAP	0.74	12,800	1.7	3.6 ± 1.1	2.7 ± 0.8
CB	1.12	13,100	1.2	3.7 ± 1.2	2.7 ± 0.8
Diploids	0.25	4,700	1.9	2.3 ± 0.6	1.8 ± 0.4

SL: shell length. SW: shell width.

Table 3.   Triploid percentages of juvenile *H. rubra* 22 weeks after settlement in 6-DMAP and CB treatments.

Treatments	No. of juveniles assessed[*]	Number of triploids	Number of diploids	Triploid (%)
6-DMAP	50	50	0	100
CB	40	33	7	82.5
Diploids[**]	20	0	20	0

[*]   Ploidy status was assessed individually.
[**]   Those diploid juveniles were used to locate the diploid peaks.

Table 4.   Average shell length and width (± SD) of triploid and diploid juvenile *H. rubra* 22 weeks after settlement in CB treatment.

Treatment	SL (mm)			SW (mm)		
	Triploids (*n* = 33)	Diploids (*n* = 7)	Difference (%)	Triploids (*n* = 33)	Diploids (*n* = 7)	Difference (%)
CB	4.2 ± 1.1	4.9 ± 1.2	17	3.1 ± 0.8	3.7 ± 0.9	19

SL: shell length. SW: shell width. Difference = (diploids – triploids)/triploids × 100 of SL or SW.
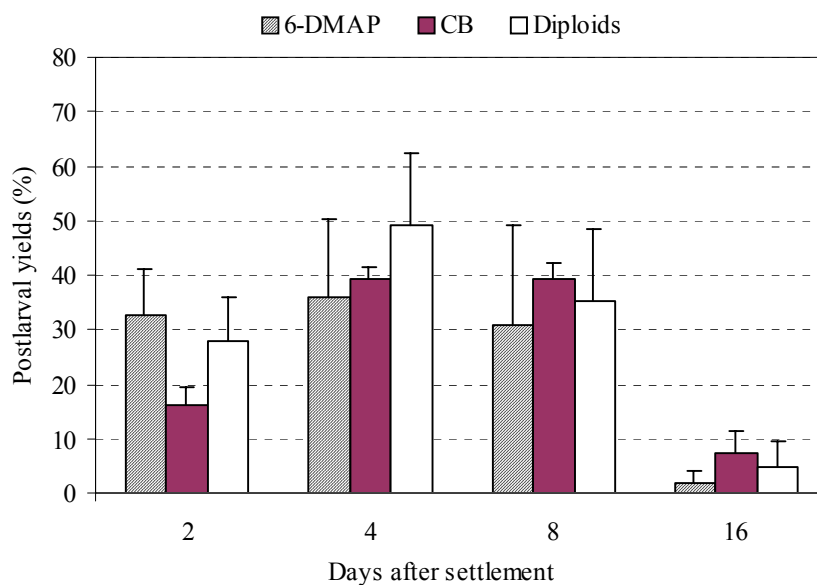
Figure 1.   Yields of post-larval (newly metamorphosed larvae) of *H. rubra* in 6-DMAP and CB treatments after settlement (*n* = 3). Error bars represent standard deviation.
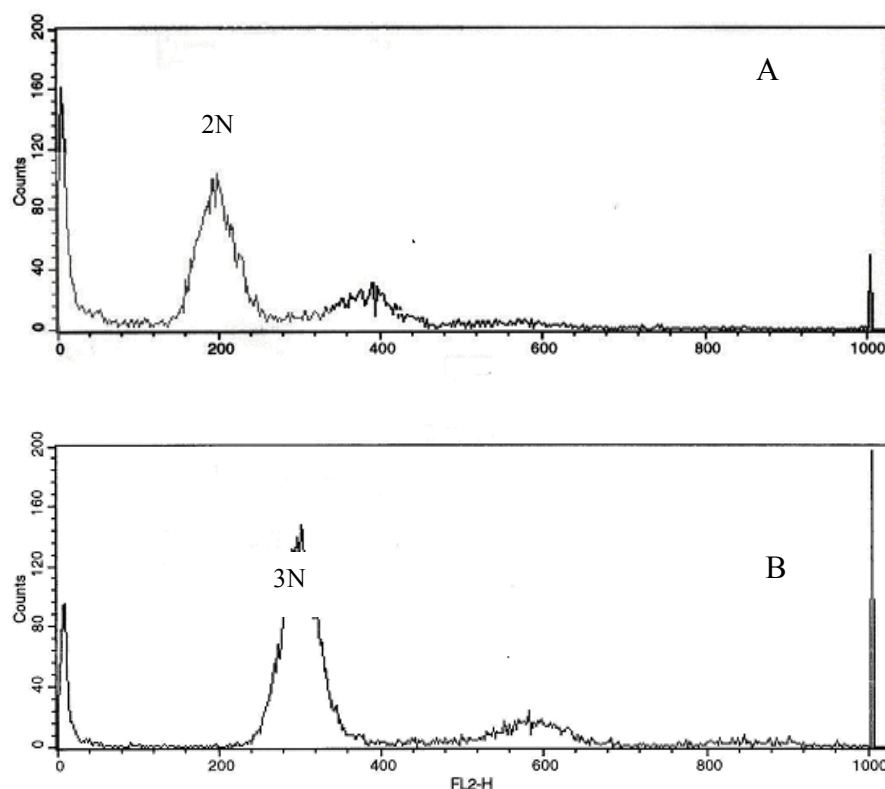
Figure 2.   Flow cytometric histograms of an individual juvenile *H. rubra* 22 weeks after settlement. A: a diploid (2N) with a peak position at 197; B: a triploid (3N) with a peak position at 297 (1.5 times the diploid). The minor peak in each histogram indicates mitotic activities. FL2-H: fluorescent density (relative DNA contents).

4.1.4. Growth and feeding in juvenile triploid and diploid blacklip abalone, *Haliotis rubra* (Leach), at two temperatures

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4.1.4.1. Abstract

Growth and feeding of juvenile triploid and diploid blacklip abalone *Haliotis rubra* (Leach, 1814) were investigated at two temperatures of 17 and 21°C over a 50-day period. There were no differences in growth between triploid and diploid abalone as measured by shell length and body weight. Both triploid and diploid abalone increased in length but not in weight at 21°C. Condition indices were similar for triploid abalone maintained at both temperatures, however, those for diploid abalone were significantly higher at 17 than at 21°C. Food intake was significantly greater yet feed conversion efficiency was significantly lower in triploid than in diploid abalone. Both the feeding parameters were independent of temperature. On average, diploid abalone were able to convert one gram of dry food ingested to 0.58 g of body weight, but triploid abalone only 0.44 g.

4.1.4.2. Introduction

Experiments on triploid molluscs have often produced encouraging results in that triploids exhibit faster growth than their diploid counterparts (Allen and Downing 1986; Eversole *et al.* 1996; Hand *et al.* 1998; Ruiz-Verdugo *et al.* 2000). It is commonly believed that the increased body size in triploids is caused by their (total or partial) sterility resulting in energy reallocation from reproduction to growth (Allen and Downing 1986; Beaumont and Fairbrother 1991; Hand *et al.* 1998); increased hetero-zygosity that would be expected to produce the heterosis effect and manifested as faster growth or higher viability (Stanley *et al.* 1984; Beaumont and Fairbrother 1991; Hawkins *et al.* 1994); or polyploidy giantism in which the increased volume of triploid cells is not compensated by a reduction of cell number (Guo and Allen 1994). There is also the possibility that triploids may have potential for faster transcription of gene products because of the presence of three copies of the same genes (Magoulas *et al.* 2000), or that growth of diploids was depressed because of partial and progressive loss of chromosomes exposing recessive deleterious mutations with a higher probability (Zouros *et al.* 1996). Moreover, the growth benefits of triploids may be attributable to their greater contribution to soft tissue production (Kesarcodi-Watson *et al.* 2001a), greater vigour due to the selection effects of treatment (induction of triploidy) (Zhang *et al.* 1998), or increased resistance to high temperatures (Fujino *et al.* 1987).

Production performance advantages of triploids over diploids may vary according to the environments such as temperature and food availability (Hand *et al.* 1998; Hand and Nell 1999). A relatively small increase in growth rate advantage was reported for triploid oysters grown at lower temperatures, but at warmer (more favourable) temperatures triploid oysters grew much faster than diploids (Hand *et al.* 1998; Nell 2002). However, little is known of the effect of food availability or of the nutritional requirements on performance of triploid molluscs. With the accumulation of evidence, it is increasingly clear (though not in through main stream in literature) that not all growing environments are conductive to superior growth performance of triploids (Davis 2004). Triploid oysters do not outperform their diploid counterparts in poor growing areas or sub-optimal environments (Nell 2002; Davis 2004). Although Nell (2002) and Davis (2004) did not define the

nature of the poor growing areas or suboptimal environments, nutrition is probably one of the factors implicated (Davis, personal communication 2004). Guo and Allen (1994) speculated that because triploid cells are larger, they might need more nutrients to grow and divide. As a result, triploids would not be larger than diploids in an environment where food supply is limiting (before diploids reach sexual maturity).

In this study, an investigation to compare the growth performance of triploid and diploid blacklip abalone *Haliotis rubra* (Section 4.1.5), sibling triploids and diploids showed similar growth during the juvenile stages. It was also of interest to test if growth of triploid and diploid abalone are differentially accelerated under favourable temperatures. During the daily maintenance, it was noticed that triploid abalone consumed more food than diploid abalone (after reaching about 30 mm shell length). This promoted an investigation of food intake and feed conversion efficiency of the two types of animals. The aim of the present study was to examine (1) growth and (2) feeding of the juvenile triploid and diploid blacklip abalone *H. rubra* under different temperature regimes.

4.1.4.3. Materials and methods

Animals

Sibling triploid and diploid *H. rubra* were produced in April 2002, with triploids induced by inhibition of the second polar body (PB2) formation using either 6-dimethylaminopurine (6-DMAP) or cytochalasin B (CB) (See Sections 4.1.1 and 4.1.2). To compare their growth, animals were divided into five replicate raceways at seven months of age from each of the two triploidy treatments and the diploid control (see Section 4.1.5).

Juvenile triploid and diploid *H. rubra* used in this experiment were those as described in Section 4.1.5, when they were 22 months old. Only triploids produced with 6-DMAP were used because of the high triploid percentage obtained (> 96% examined at 19 months of age). A random sample of 24 abalone was collected from each raceway (a total of 120 animals for each ploidy class), and their shell length and body weight measured. After a two-way nested ANOVA (replicate raceway nested within ploidy) confirmed that there were no significant differences in length and weight ($P =$ 0.30 and 0.17 respectively) among replicate raceways, all the individuals from each ploidy class were pooled. These constituted the triploid and diploid populations, respectively, of this experiment. This minimized potential tank effects, which may be build up during the long grow-out period and thus confound results if the triploid and diploid abalone had each been chosen from a single rearing unit.

Experimental conditions

Growth and feeding of triploid and diploid *H. rubra* were compared at two temperatures of 17 and 21°C (means ± SD of 16.8 ± 0.2 and 20.6 ± 0.2°C, respectively, for 50 measurements). This constituted four treatments (two triploidy classes × two temperatures). A temperature of 17 °C was chosen because it was the optimal temperature for growth of *H. rubra* (> 30 mm shell length) (Gilroy and Edwards 1998; Heasman *et al.* 2004), and also approximated the average of the sea temperatures for the study site in winter (Section 4.1.5). In keeping with the temperature range at the study site, a warmer but not stressful temperature of 21°C (juvenile *H. rubra* show reduced feeding activities at temperatures > 22°C, personal observations) was also tested.

Six rearing containers, each stocked with ten abalone randomly selected from each ploidy population, were used in each temperature treatment. The containers had a base area of 36 × 22 cm with water depths maintained at 9 – 10 cm using a standpipe. Each was provided with fresh seawater filtered to 10 μm at a flow rate of 0.25 – 0.30 L min^{-1} and constant aeration. A concrete brick (22 × 11 × 3 cm) with simple legs was placed in each container to provide a 2-cm high cavity

that served as a diurnal shelter for the abalone, The containers were randomly positioned in an air-conditioned room provided with a photoperiod regime of 16 h light and 8 h dark.

Abalone were held in the containers for five days at an ambient seawater temperature of about 22°C to acclimate to their new surroundings. During this time they were individually tagged and three mortalities replaced. They were then acclimated to allocated temperatures at a rate of 1°C per day. Food was offered during the period of temperature acclimation. The experiment was commenced three days after the lower temperature of 17°C was reached and lasted for 50 days.

Growth parameters

Shell length and body weight were recorded for each animal at the start and the end of the growth period (Table 1). Length was measured to the nearest 0.1 mm using a vernier calliper, and weight to 0.01 g with a digital balance. Relative growth rate (RGR) over the entire study period was calculated for each individual as:

$$\text{RGR (length or weight) (\%)} = \text{(final – initial)/initial measurement} \times 100$$

Condition index (CI) was determined for the animals at the start and the end of the study as:

$$\text{CI} = \text{soft tissue weight/body weight} \times 100$$

where soft tissue weight = body weight – shell weight. Shell weight was estimated using the following relations (unpublished data):

$$\text{Shell weight} = 0.000121 \times \text{SL}^{2.748} \ (r^2 = 0.916, n = 40) \text{ for triploids, and}$$
$$\text{Shell weight} = 0.000137 \times \text{SL}^{2.728} \ (r^2 = 0.927, n = 40) \text{ for diploids}$$

where SL = shell length (within the range of 32 – 50 mm).

Feeding and determination of food intake

Animals were fed an artificial diet (Adam and Amos Abalone Foods, South Australia, Australia) every other day. The diet was kept in an airtight jar and stored in refrigerator. A known amount of food (1.92 – 2.59 g with water content of 10.9 – 11.7%) was placed to each of the containers after they were drained and flushed with isothermal seawater. Uneaten food was siphoned out in the following morning after about a 14-hour feeding time, collected onto filter paper using a vacuum flask, and stored frozen for later determination of dry matter. A total of 25 feeds were provided throughout the study. Food consumed by abalone per replicate container over the whole study period was estimated as:

$$\text{FI (g)} = \text{FO – FU/WS} \qquad \text{(Jackson } et\ al. \text{ 2001)}$$

where FI = dry food intake, FO = dry food offered, FU = dry food uneaten, and WS = water stability of the diet (= dry weight of the food after/before being immersed in seawater for about 14 hour at either temperature, under those identical experimental conditions without abalone). Water content and water stability of the diet were measured for five times during the study period, and the means were used for calculation of food intake. For the measurement of dry matter, all the samples were dried at 106°C for 18 – 20 h and cooled in desiccators at room temperature before being weighed. It should be noted that the calculation of food intake adopted in the present study implies that the food was taken by abalone at the time when it is offered (i.e., the food had not lost any mass before being taken), and therefore food intake is likely to be overestimated.

In contrast, the calculation of Uki and Watanabe (1992), which is equivalent to FI = FO × WS – FU, presumes that the food was taken by abalone until the time when uneaten food was collected (i.e., the food had suffered loss of weight in seawater before being taken), and therefore food intake may be underestimated. However, there is no reason to suspect that (potential) over or under-estimation of the true values of FI varied between treatments. Therefore, tests of treatment effects should still be valid and reliable, regardless of which formula is used. Feed conversion efficiency (FCE) over the study period was calculated on the basis of each container as:

$$\textbf{FCE (g/g) = BW gain/FI}$$

where BW = body weight and FI = dry food intake.

Statistical analysis

A three-way nested ANOVA, in which containers (random treatment factor) were nested within ploidy (fixed) and temperature (fixed) with individual abalone recognised as the replicate units, was applied to test initial and final size, growth and condition index of the animals. Individual data within each treatment were pooled to pursue a further analysis (two-way fixed ANOVA) in order to improve the test capacity if there was no variation among replicate containers ($P > 0.25$) (Underwood 1997; Quinn and Keough 2002).

To simplify data presentation, container means (mean of the ten individual abalone per container) were taken to plot the means and errors for each treatment if individual observations within the treatment could not be pooled. A two-way fixed ANOVA was used to examine effects of ploidy and temperature on food intake and feed conversion efficiency, with replicate containers identified as the replicate units. A Tukey HSD (Honestly Significant Difference) multiple range test was done to compare differences between treatments only when a significant interaction effect of ploidy and temperature was detected. Homogeneity of variances of the data was checked by Bartlett's test. Growth (length) was transformed to the square-root of $(X + 0.5)$, where X is the original data, to remove heterogeneity of variance. Statistical significance was accepted at $P < 0.05$. All analyses were performed using the statistical package *Statgraphics Plus for Windows 4.1* (Manugistics Inc, USA).

4.1.4.4.   Results

Growth

Initial and final sizes of the animals are shown in Table 1. Triploid abalone had a similar shell length ($F_{1, 236} = 2.04$, $P = 0.15$) but a significantly greater body weight ($F_{1, 236} = 8.17$, $P = 0.0047$) than diploid abalone at the start of the experiment. The pattern of difference remained the same at the final measurement. No mortality occurred during the study period.

Relative growth rate for shell length was 6.0 and 5.3% at 17°C, and 6.9 and 6.5% at 21°C, for triploid and diploid abalone respectively (Figure 1). Triploids tended to show a higher growth rate than diploids at either temperature, but the difference was not significant ($F_{1, 20} = 1.96$, $P = 0.18$). Both triploids and diploids grew significantly faster ($F_{1, 20} = 4.48$; $P = 0.047$) at 21 than at 17°C. The effect of temperature did not change with ploidy ($F_{1, 20} = 0.28$, $P = 0.60$). In addition, variations among replicate containers were large ($F_{20, 216} = 1.65$, $P = 0.043$). So was the individual growth within containers. The standard deviation was 17 – 100% of the mean across all the replicate containers.

Relative growth rate in body weight was 12.8 and 15.6% at 17°C, and 17.6 and 17.4% at 21°C, for triploid and diploid abalone respectively (Figure 2). In contrast to length, body weight growth of

triploids was lower than that of diploids at 17°C. However, ANOVA revealed that ploidy ($F_{1, 20}$ = 0.53; P = 0.48), temperature ($F_{1, 20}$ = 1.70; P = 0.21) and their interaction ($F_{1, 20}$ = 0.69; P = 0.42) all had no significant effects on body weight. As for length, container effect on weight was significant ($F_{20, 216}$ = 1.98; P = 0.009), and variation of individual growth within container was large with a standard deviation ranging from 30 to 143% of its mean. It should be noted that variances were not homogenous (P = 0.013) in this analysis (no transformation was possible for the data). This violates the assumption of equal variances underlying the analysis of variance and may invalidate the statistical tests. However, a conclusion that there were no significant differences among treatments is valid under the circumstance of heterogeneous variances because heterogeneity of variances leads to increased probability of Type I error (Underwood 1997).

At the onset of the study (Table 1), triploid abalone had a significantly higher condition index than diploid abalone ($F_{1, 236}$ = 119.62, P < 0.000). At the end of the study, a significant interaction effect of ploidy and temperature was detected ($F_{1, 236}$ = 5.58, P = 0.017). A Tukey HSD multiple range test found that condition index was still significantly higher (P < 0.05) in triploids than in diploids, however, it was also significantly greater for diploids at 17 than at 21°C (Table 1).

Feeding

Triploid abalone had significantly higher food intake than their diploid counterparts ($F_{1, 20}$ = 38.80, P < 0.000), food consumption being 27.1 and 29.7 g for triploid abalone compared to 22.0 and 22.3 g for diploid abalone at 17 and 21°C respectively (Figure 3). However, triploid abalone were unable to assimilate food as efficiently as sibling diploids ($F_{1, 20}$ = 4.98, P = 0.037). For every gram of dry food ingested, diploid abalone at 17°C were able to convert to an average of 0.59 g of body-weight, but triploid abalone only 0.40 g. The corresponding body-weight conversion for diploid and triploid abalone at 21°C was 0.56 and 0.47 g respectively (Figure 4). The effect of temperature and interaction of ploidy and temperature was not significant (P > 0.05) for either feeding parameter measured.

4.1.4.5. Discussion

Growth

Individual growth of abalone within cultured populations is highly variable (Mgaya and Mercer 1995; Preece and Mladenov 1999; Huchette *et al*. 2003). This was also the case in the present study in which individual growth rates, as measured by the relative increase of shell length and body weight, varied greatly for triploid and diploid abalone both within and among replicate containers (within treatments). The observed variations, on the other hand, may also be attributable to the initial pooling of animals from different culture raceways, the small sample size (a group of ten abalone may not be a representative sample size as a result of the large individual variation), and the intrinsic variation among culture units.

Ploidy was found to have no significant effect on growth of juvenile *H. rubra*. Further, the sibling triploid and diploid abalone used in the present study showed similar growth during the grow-out period (Section 4.5). These results are in agreement with the common observation that growth of triploid molluscs in general does not differ much from that of diploids during juvenile stages or before sexual maturation (Eversole *et al*. 1996; Hand *et al*. 1998; Ruiz-Verdugo *et al*. 2000).

Growth in length was significantly accelerated at 21°C for both triploid and diploid abalone. However, a commensurate increase in weight did not occur. Although condition index remained similar in triploid abalone maintained at 17 and 21°C at the end of the study, diploid abalone had a significantly higher condition index at 17 than at 21°C (Table1). This suggests that diploid (but not triploid) abalone invest more in soft tissue rather than in shell growth at 17°C or the reverse at 21°C. Shpigel *et al.* (1992) found that growth rates (weight) increased while dry tissue and

condition index decreased for both triploid and diploid Pacific oyster Crassostrea gigas at an elevated (stressful) temperature of 30°C. They attributed the increase in growth to an increase in shell growth relative to tissue growth. Such an account may, however, not be precise because animals used in that study, in particular the diploid oysters, spawned (Shpigel *et al.* 1992).

Although the present study was not aimed at investigating the optimal temperature for growth of *H. rubra*, the results obtained, as far as length is concerned (faster growth at the higher temperature of 21°C), are not in accordance with those of Gilroy and Edwards (1998) and Heasman *et al.* (2004), who found that optimal temperature for growth of *H. rubra* (> 30 mm shell length) was 17°C. It is acknowledged that these fundamentally conflicting results are perplexing and could be attributable to the fact that the alternation of feeding and non feeding days applied to the present experiment (food was only available 50% of the time), might have inadvertently imposed serious constraints to rates of food ingestion, assimilation and growth thereby seriously confounded results and undermining their practical significance.

Feeding

The (overall) consumption and utilisation of food are rarely quantified in triploid molluscs probably because most of the species studied so far are the filter-feeding bivalves, culture of which in the field does not incur food costs. In the present study, triploid abalone were found to have higher food intake but lower feed conversion efficiency than diploid abalone. Food intake is related to body weight in (diploid) juvenile abalone (Farías *et al.* 2003). Although triploid abalone used in the present study had a greater body weight than the control diploids (Table 1), the actual margin was < 9% for both initial and final weight. Therefore, size difference may probably not play a major role in the observed difference in food intake between triploid and diploid abalone. In the studies on the physiological energetics on molluscan bivalves, Kesarcodi-Waston *et al.* (2001a, b) found that triploid Sydney rock oysters Saccostrea commercialis had similar or lower clearance rates than diploids, while Manson *et al.* (1988) reported similar but higher filtration rates in triploids than in diploids for soft-shelled clam *Mya arenaria*. However, it is difficult to compare these food consumption results with that of the present study due to the differences in methodology of research and in feeding behaviour of the animals (i.e., hours vs. days of feeding time; and filter- vs. graze-feeding). Feed conversion efficiency obtained was 0.40 – 0.47 for triploid abalone and 0.56 – 0.59 for diploid abalone, both of which fall within the range of 0.3 – 1.3 for other (diploid) temperate abalone (Fleming *et al.* 1996).

The feed conversion efficiency in triploid abalone was significantly lower. This was because triploid abalone had a higher feed intake than but similar growth rates to diploid abalone, indicating that triploid abalone required greater quantity of food to maintain basic metabolic process. The practical implication of this finding is that juvenile triploid abalone may not be able to perform as well as diploids if food supply is limited. However, food availability should not be an issue of concern in abalone aquaculture because animals are fed artificially to excess. A physiological energetic study (Navarro *et al.* 2002; Farías *et al.* 2003) would help understand whether this disadvantage in utilisation of food in triploid abalone is associated with a lower absorption (assimilation) efficiency, or a higher energy expenditure for metabolism (respiration and excretion) and mucus secretion (in association with movement). It has been shown that there were no differences in the overall physiological energetics between triploids and diploids in several species of bivalves, e.g., the soft-shelled clam M. arenaria (Mason *et al.* 1988), the Manila clam *Tapes philippinarum* (Laing and Utting 1994) and the Sydney rock oyster *S. commercialis* (Kesarcodi-Waston *et al.* 2001b). Nevertheless, triploid and diploid abalone used in the present study had recently begun to form gonads and it appeared that gonadal development was more advanced in triploids than in diploids (on the visual scales, 45% of the triploid individuals had formed a brow-yellow gonad on the surface of conical appendage at the onset of the present study, while only 21% of the diploid individuals developed patchy gonads, Section 4.5).

It is likely that costs (to produce) per unit of mass of reproductive tissue are larger than those of somatic tissues, especially during the early stage of gametogenesis (Honkoop 2003). Therefore, the lower feed conversion efficiency obtained in triploid abalone may also be due to the fact that triploid and diploid abalone were not at the same ontogenetic stage although they were derived from a same batch of fertilised eggs. In fish (Atlantic salmon), lower or equal food conversion efficiency has been observed in triploids compared to diploids (Sunde *et al.* 2001; Oppedal *et al.* 2003). Nevertheless, food intake and feed conversion efficiency may be affected by temperature (Fleming *et al.* 1996; Heasman *et al.* 2004). That both the two feeding parameters were independent of temperature in the present study is presumably because of the narrow range between the two experimental temperatures adopted.

In summary, growth was similar between juvenile triploid and diploid *H. rubra*. However, the maintenance of triploid abalone may be more costly because of the lower feed conversion efficiency in triploid than in diploid abalone. A consequence is that triploids, rather than being an advantage, may be a less efficient tool in commercial *H. rubra* aquaculture.

Notwithstanding the above discussion, it is acknowledged that results of this study in relation to relative feeding and growth performances of sibling triploid and diploid *H. rubra* are not generally consistent with those reported for other molluscs. As already stated above, this could be attributable to the fact that the alternation of feeding and non feeding days applied here, might have inadvertently imposed serious constraints to rates of food ingestion, assimilation and growth thereby seriously compromising the results, their interpretation but most importantly, their practical significance.

4.1.4.6.   References

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Table 1.   Initial and final shell length (SL), body weight (BW) and condition index (CI) (means ± SE, $n = 60$) of triploid (3N) and diploid (2N) *H. rubra* exposed to two temperatures over a 50-day period.

Treatments		Initial			Final		
		SL (mm)	BW (g)[1]	CI (%)[1]	SL (mm)	BW (g)[1]	CI (%)[2]
17°C	3N	37.3 ± 0.38	9.11 ± 0.26	71.8 ± 0.26	39.5 ± 0.38	10.23 ± 0.28	70.6 ± 0.22[a]
	2N	36.8 ± 0.38	8.38 ± 0.25	68.8 ± 0.26	38.7 ± 0.42	9.69 ± 0.32	69.1 ± 0.28[b]
21°C	3N	37.3 ± 0.40	8.97 ± 0.28	71.3 ± 0.26	39.8 ± 0.39	10.36 ± 0.29	70.4 ± 0.21[a]
	2N	36.7 ± 0.36	8.22 ± 0.24	68.5 ± 0.27	39.1 ± 0.39	9.51 ± 0.28	67.8 ± 0.23[c]

[1] Within a column, values for triploid abalone were significantly larger ($P < 0.05$) than diploid abalone as confirmed by a two-way fixed ANOVA test.

[2] Within a column, means sharing a same superscript did not differ significantly ($P > 0.05$) as confirmed by a Tukey HSD multiple range test.
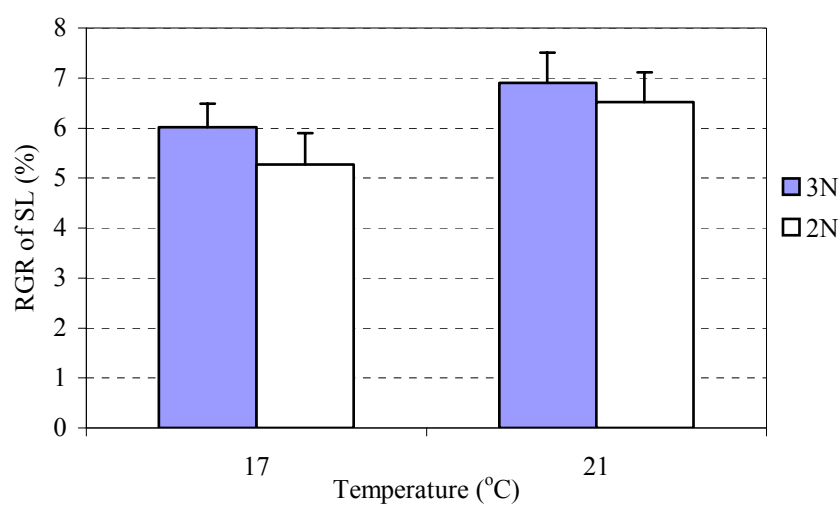
Figure 1. Relative growth rate (RGR) of shell length (SL) in triploid (3N) and diploid (2N) *H. rubra* exposed to two temperatures over a 50-day period. Error bars represent standard errors of the container means ($n = 6$).
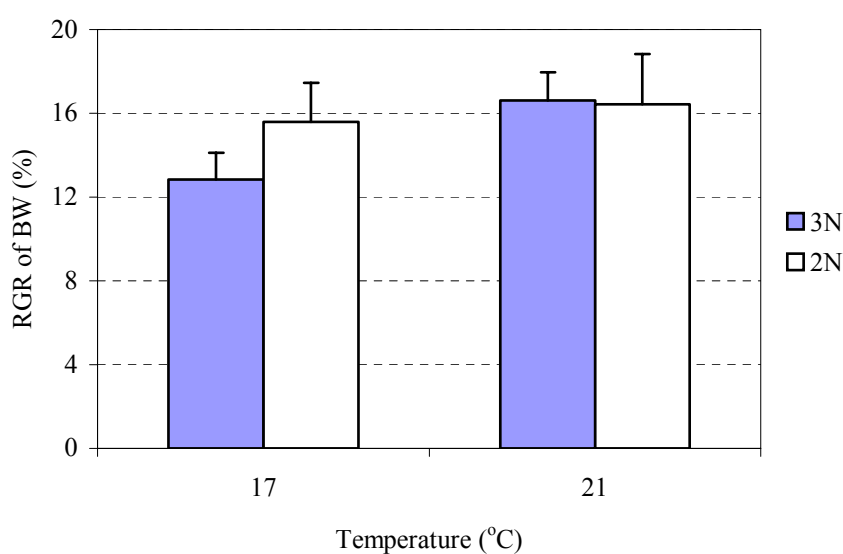
Figure 2. Relative growth rate (RGR) of body weight (BW) in triploid (3N) and diploid (2N) *H. rubra* exposed to two temperatures over a 50-day period. Error bars represent standard errors of the container means ($n = 6$).
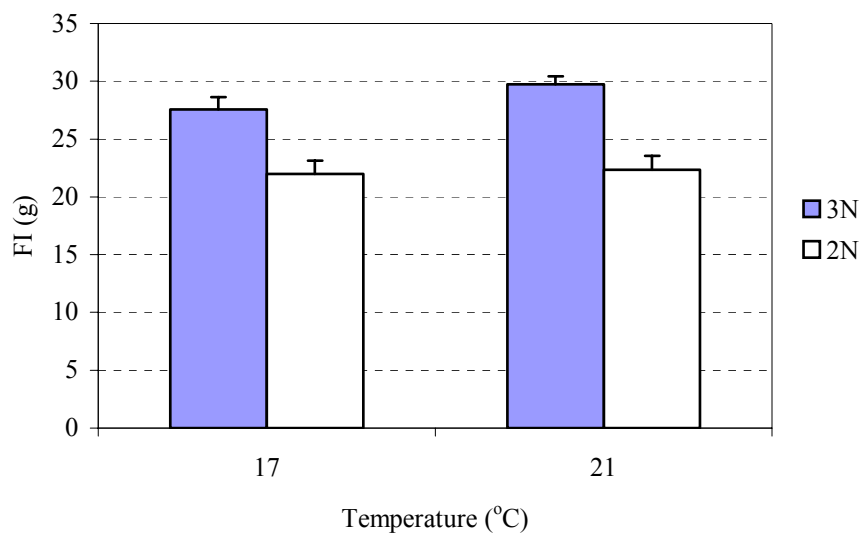
Figure 3. Dry food intake (FI) of triploid (3N) and diploid (2N) *H. rubra* exposed to two temperatures over a 50-day period. Error bars represent standard errors (*n* = 6).
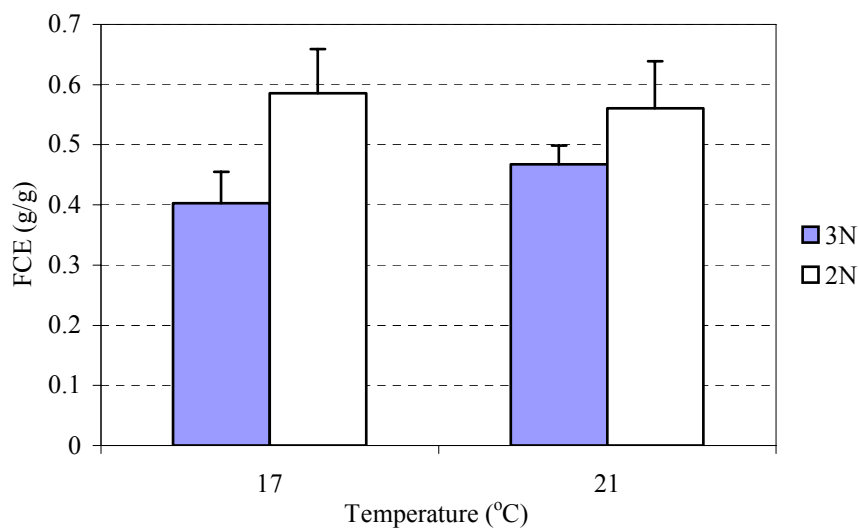
Figure 4. Feed conversion efficiency (FCE) of triploid (3N) and diploid (2N) *H. rubra* exposed to two temperatures over a 50-day period. Error bars represent standard errors (*n* = 6).

4.1.5.   Growth and reproductive performance of triploid and diploid blacklip abalone, *Haliotis rubra* (Leach)

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4.1.5.1.   Abstract

Growth and reproduction of triploid and diploid blacklip abalone *Haliotis rubra* (Leach, 1814) were compared in a two-year study. Triploidy was induced by inhibition of the second polar body formation using 6-dimethylaminopurine (6-DMAP) or cytochalasin B (CB). There were no differences in shell length, body weight, condition index and survivorship between triploid (treatment) and control diploid abalone. However, triploid abalone had a more elongated shell and a higher foot muscle index (in 6-DMAP treatment only) than diploid abalone. A slightly curvilinear growth in shell length was conformed to all treatments. While diploid abalone had reached sexual maturity and spawned by the end of the study, gonadal development and gamete maturation were abnormal in triploids compared to diploids. Female triploids lacked an apparent gonad at the macroscopic level but microscopic examination revealed that they had a thin layer of oogonia development. In contrast, male triploids were able to form similar sized gonads to diploids, but with brown-yellow discolouration and stalled gametogenesis at spermatocyte formation. Sex ratio of triploid abalone did not deviate from 1:1. Growth and gonadal maturation occurred concurrently in diploid abalone, and there was no indication that growth of (diploid) abalone slowed down with the onset of sexual maturation.

4.1.5.2.   Introduction

Chromosome set manipulation in molluscs has received wide attention in the last two decades. Research has primarily focused on the induction and evaluation of triploidy in bivalve species of commercial importance (Beaumont and Fairbrother 1991; Nell 2002). The principal value of triploids for aquaculture arises from their sterility, presumably because of failure in synapse of three sets of chromosomes during meiosis. Sterility may lead to faster growth of triploids owing to energy reallocation from reproduction to somatic growth. Sterility may also result in better meat quality of triploids in association with reduced spawning activities (Beaumont and Fairbrother 1991).

Increased body size for triploids has been reported, with few exceptions (Mason *et al.* 1988; Matsuda and Yamakawa 1993; Davis 1997; Maldonado-Amparo *et al.* 2004), for most bivalve species examined so far, including the clams *Mercenaria mercenaria* (Eversole *et al.* 1996) and *Mulinia lateralis* (Guo and Allen 1994b); the oysters *Crassostrea gigas* (Allen and Downing 1986, 1990; Akashige and Fushimi 1992), *C. virginica* (Stanley *et al.* 1984; Barber and Mann 1991) and *Saccostrea commercialis* (Nell *et al.* 1994; Cox *et al.* 1996; Hand *et al.* 1998); the pearl oyster *Pinctada martensii* (Jiang *et al.* 1993); and the scallops *Argopecten irradians* (Tarbarini 1984), *A. ventricosus* (Ruiz-Verdugo *et al.* 2000) and *Chlamys nobilis* (Komaru and Wada 1989).

However, complete sterility has not been conferred as a general rule on triploid molluscs. Rather, retarded gonadal development or abnormal gametogenesis has been common observations (Beaumont and Fairbrother 1991). Female triploid bivalves typically produce immature oocytes,

development of which is arrested at the pre-vitellogenic stage. In male triploids, such as *C. nobilis* (Komaru and Wada 1989), *C. virginica* (Barber and Mann 1991), *M. mercenaria* (Eversole *et al.* 1996) and *S. commercialis* (Cox *et al.* 1996), spermatocytes are usually formed but not spermatids or spermatozoa. In contrast, some other triploid bivalves including *C. gigas* (Guo and Allen 1994a) and *P. martensii* (He *et al.* 2000) are able to produce viable (fertilisable) gametes, but generally in greatly reduced quantities compared to diploids. The combined effects of enhanced growth and sterility (or reduced reproductive output) may therefore offer triploids useful tools of increasing commercial benefits and of improving marketability in mollusc aquaculture. This is well illustrated by considerably reduced time to reach market size and extended marketable condition seasons for the triploid oysters *S. commercialis* (Nell *et al.* 1994; Hand *et al.* 1998).

In this study, growth and reproductive performance of triploids relative to diploids was examined in the blacklip abalone *Haliotis rubra* (Leach, 1814) (Gastropoda). The potential application of triploidy for aquaculture was assessed in this species as well.

4.1.5.3.   Materials and methods

Animals

A large batch of sibling triploid and diploid *H. rubra* was produced in April 2002 from multiple parents comprising seven females and four males. Triploidy was induced by inhibition of the second polar body (PB2) formation using 6-dimethylaminopurine (6-DMAP) or cytochalasin B (CB). Percent triploidy was 100% for 6-DMAP treatment and 82.5% for CB treatment when examined 22 weeks after fertilisation by flow cytometry (Liu *et al.* 2004). Animals were grown in outdoor nurseries and reached a mean shell length of 8.1 – 9.4 mm (seven months old) (Table 1) before being deployed in this study to compare their growth and reproductive performance.

Experimental conditions

The present study was conducted at the Abalone Research Unit (32^o43'S, 152^o11'E) of Port Stephens Fisheries Centre, NSW Department of Primary Industry. Five replicate raceways, each comprising a random sample of 200 individuals, were applied to each triploidy treatment and the diploid control. Initial size of the animals was recorded for each treatment from a different sub-sample of 60 individuals. Small raceways with base areas of 36×22 cm and water depths maintained at 9 – 10 cm using a standpipe were used for the first six months of culture, and larger ones with base areas of 60×40 cm and water depths of 17 – 18 cm used thereafter. The small raceways were each equipped with two concrete bricks ($22 \times 11 \times 3$ cm) with two legs providing a 2-cm high cavity underneath that provided diurnal shelters for abalone. The larger raceways (Plate 1) were equipped with two $40 \times 19 \times 4$ cm shelters with two legs providing 5-cm high cavities underneath. The raceways were randomly positioned on outdoor benches under a weatherproof canopy. They were provided with ambient seawater at a flow rate of 2 – 3 L min^{-1} and constant moderate aeration. Mean monthly seawater temperature of the study site varied in the range of 17 – 23^oC (Figure 1).

Abalone were fed ad libitum with a commercial diet (Adam and Amos Abalone Foods, South Australia, Australia) every day, and uneaten food and faeces flushed out each morning after the raceways were drained. The raceway walls were also cleaned routinely to prevent an accumulation of benthic or sessile organisms. At the time of writing of this report the abalone had been cultured for two years from 7 to 31 months of age (December 2002-December 2004). During this time stocking density was adjusted twice by culling a small number of abalone from each raceway depending on the mortality. These procedures both reduced stocking density and maintained similar densities among replicates.

Ploidy determination

Percent triploidy was re-verified when animals were 19 months old with the double freezing methods (Hand 2002), from 30 individuals in 6-DMAP treatment and 35 in CB treatment. Briefly, a small piece of gill tissue from an individual abalone was inserted into a 1.75 micro-tube filled with marine phosphate buffered saline (MPBS) (Allen 1983) containing 10% dimethylsulfoxide (DMSO) and then frozen at -80°C. Samples were thawed at room temperature and centrifuged at 2000 rpm for 5 min. After removal of supernatant, 0.65 propidium iodide (PI) staining solution (Allen 1994) was added into each micro-tube and cell pellets were re-suspended by gentle mixing and stained for 1 h at room temperature. Samples were stored at -80°C, thawed and filtered through a 35 μm-mesh immediately before being analysed by flow cytometry.

Sampling

Samples were taken at three-month intervals after initial stocking (i.e., at 10, 13, 16, 19, 22, 25, 28 and 31 months of age). Between 8 and 15 individuals were removed from each raceway and measured for shell length and body weight, and 4 – 5 of them for dry weight. The destructive sampling strategy avoided confounding effects of handling on growth and survival, and also helped reduce culture density over time. Dry weight was obtained with samples dried at 106°C for 1 – 820 h and cooled in desiccators at room temperature before weighing. Shell length was measured to the nearest 0.1 mm using vernier callipers and body weight to 0.1 g with a digital balance. Condition index (CI) was defined as:

$$\textbf{CI = dry soft tissue weight/dry body weight} \times \textbf{100}$$

Foot muscle index (FMI) was recorded after control diploid abalone had reached sexual maturation and determined as:

$$\textbf{FMI = dry foot muscle weight/dry body weight} \times \textbf{100}$$

Gonadal development

Gonadal development was examined both macroscopically and microscopically. A mature gonad is strongly coloured ranging from either green to reddish brown (females) or cream/greyish white (males). This allows mature (diploid) abalone to be sexed easily by visual inspection (Hahn 1989). For histological examination, only high percentage triploid abalone produced with 6-DMAP and control diploids were continuously monitored. Two abalone were taken from each raceway on each sampling occasion. Conical appendages (gonads) were excised and fixed in Davidson's fixative (Appendix 1) (4°C) for 24 hours and preserved in 70% ethanol. Samples were embedded in paraffin (Appendix 2), sectioned at 5 – 7 μm across the midpoint of the conical appendage, and stained with haematoxylin and eosin (H&E) (Appendix 3) after deparaffination (Appendix 4). Gonad area (GA) was estimated by assuming that the sectioned areas were round or elliptical in accordance with the following formulae:

$$\textbf{GA = total area of section – area of digestive gland}$$

Gonad index (GI) was defined as:

$$\textbf{GI = gonad area (GA)/total area of section} \times \textbf{100} \text{ (Shepherd and Helene 1974)}$$

Mortality

Mortality (M) of abalone was monitored every three-months for each raceway and expressed as:

M = dead/initial number of abalone of a three-month period × 100

Statistical analysis

Because repeated sampling of the same experimental units (raceways) raises the issue of non-independence of data, which violates one of the assumptions of analysis of variance (Underwood 1997), a formal statistical test was done only for the last set of data collected at 31 months of age. Two-way nested ANOVAs were used to analyse the effect of ploidy on shell length, body weight, shell length-width ratio, condition index and foot muscle index, with raceway (random) nested in ploidy (fixed). Individual data within treatment were pooled to pursue a further analysis (one-way fixed ANOVA) to improve test capacity if there were no variations among replicate raceways ($P > 0.25$) (Underwood 1997; Quinn and Keough 2002). Abalone in CB treatment contained a mixture of triploids (CB-3N) and diploids (CB-2N). However, they were not separated for the statistical purposes because to do so data would not be balanced among replicate raceways thereby invaliding the statistical tests of a nested ANOVA (Underwood 1997). Instead, size ranges of the animals are presented for comparison.

A one-way fixed ANOVA was applied to examine the effect of ploidy on gonad area and gonad index. It should be noted that viability among replicate raceways was not measured in this analysis owing to the small number of specimens available (two from each raceway), and that triploid abalone refer to those produced with 6-DMAP and exclusively comprised males because females only had a minor degree of gonadal development (see Section Visual examination of gonads). Tukey HSD (Honestly Significant Difference) multiple range tests were used to compare differences between means. Homogeneity of variances of the data was checked by Bartlett's test. All statistical significances were set at $P < 0.05$. To simplify data presentation, raceway means (mean of the individual data of a container) were taken to plot means and standard errors for each treatment if individuals within treatments could not be pooled ($P < 0.25$). The analyses were performed using the statistical package Statgraphics Plus for Windows 4.1 (Manugistics Inc, USA).

Relationships between shell length and body weight of abalone were analysed using Microsoft® Excel 2000 after individual data (8 – 10 abalone from each container at each chosen time), within treatments collected over time, were pooled.

4.1.5.4.   Results

Percentage of triploidy

Percent triploidy determined by flow cytometry in 19 months old abalone was 97% in 6-DMAP treatment. Ploidy status of only one of the 30 individuals assessed was in doubt and this was probably due to technical failure of the assay procedure. By contrast, only 60% of CB treated abalone was confirmed as triploids.

Ploidy levels were further examined by visual confirmation based on the unique gonadal patterns of triploid and diploid abalone (see Section Visual examination of gonads). This resulted in a triploid percentage of 100% and 46% for the 6-DMAP and CB treatments, respectively, at 31 months of age.

Growth

Shell growth was very similar across all treatments including the diploid control especially during the first 18 months of grow-out (Figure 2). At the final sampling reported here (31 months of age), mean shell lengths for the 6-DMAP and CB treatments and the control were 59.1, 60.9 and 59.2 mm respectively (Table 1), and no significant differences were detected ($F_{2,\,117}$ = 1.16, P = 0.32). A slightly curvilinear growth was conformed to all treatments and the growth curve was best described (for combined data of all treatments because of similar growth) by a relationship of SL (mm) = $0.0230X^2 + 1.2192X – 0.4128$ (r^2 = 0.999) (where SL = mean shell length and X = months post fertilisation). In accordance with this growth, shell increments were higher in the second year (28 – 30 mm gained from 19 to 31 months of age) than in the first year of culture (22 mm gained between 7 and 19 months of age).

As with shell length, growth in body weight did not differ significantly with treatment during most of the grow-out period (Figure 3). Although an apparent divergence in mean body weight appeared at 28 months of age, no difference ($F_{2,\,117}$ = 1.18, P = 0.31) in body weight was found at 28 months of age (Table 1). Growth in body weight was best described (for combined data of all treatments because of similar growth) by a relationship of BW (g) = $0.002445X^3 – 0.050062X^2 + 0.359191X – 0.665805$ (r^2 = 0.998) (where BW = body weight and X = months post fertilisation). Nonetheless, mean body weight of diploid abalone in CB treatment (CB-2N) was greater than that of triploids from both the CB (CB-3N) and 6-DMAP treatments and control diploids (Table 1). A greater body size for CB-2N was especially evident at 28 months of age (data not shown).

Length-weight relationships

Body weight (BW) was found to increase as the approximate cube of shell length (SL) for abalone in both the 6-DMAP and CB treatments and the diploid control (Figure 4). Best-fit equations (n = 380) were BW = $0.000085 \times SL^{3.183}$ (r^2 = 0.993) for 6-DMAP triploid abalone, BW = $0.000086 \times SL^{3.163}$ (r^2 = 0.994) for CB triploid abalone, and BW = $0.000090 \times SL^{3.190}$ (r^2 = 0.993) for diploid abalone. The cubic relationship of shell length to body weight is indicative of allometric growth. Allometric relationships are equivalent to an alternative linear relationship for the log-transformed data. ANCOVA analysis of the log transformed data revealed significant differences (P < 0.01) in slopes of the regression lines among the three treatments, implying that the three groups differ significantly in morphometry and hence morphology.

Morphology

The most apparent variation in morphology was that triploid abalone had a more elongated shell than diploid abalone. This was reflected by a significantly higher (*P* < 0.01) shell length-width ratio for 6-DMAP triploids than for control diploids (Figure 5). Shell length-width ratio of abalone in CB treatment was also significantly different (*P* < 0.01) from that of control diploid abalone. It was also found that triploids in the CB treatment (CB-3N) had a similar shell length-width ratio (1.42 ± 0.01, means ± SE, *n* = 18) to 6-DMAP triploids, and this ratio in diploids in the CB treatment (CB-2N) (1.34 ± 0.01, *n* = 22) was similar to that of control diploids.

Condition and foot muscle indices

There were no general trends in condition indices for the triploidy treatment and the diploid control over the 24-month duration of the study (Figure 6). Soft tissues varied over the range of 29 – 34% of the total (dry) body weight.

Foot muscle index value was higher in 6-DMAP triploids than in control diploids after the latter had reached sexual maturity. However, a greater foot muscle index value was found only for abalone in CB treatment at 28 months of age (Figure 7). Also, there was no consistent pattern in

foot muscle index value for either triploid (CB-3N) or diploid abalone (CB-2N) in the CB treatment on the two sampling occasions (data not shown).

Mortality

Mortality was low (< 2%) and similar for triploid and diploid abalone over the two-year study, and no consistent patterns of variation were apparent (Figure 8).

Visual examination of gonads

Patchy looking gonadal tissues were observed on the surface of conical appendages in a small number (10 – 21%) of diploid abalone at 19 and 22 months of age. By 25 months of age however, all diploid abalone had developed a large mature gonad that was reddish in colour for females and cream-white for males (Plate 2B,D). The gonads enlarged as animals grew, as shown by both steadily increased gonad areas and gonad area index values at 22 to 31 months of age (Figures 9 and 10). Data of female and male diploid abalone were pooled after a preliminary test revealed no difference between them. This was in spite of the observation that some diploid abalone in two of the five replicate raceways had spontaneously spawned just days before the final sampling.

Gonads of triploid abalone in the 6-DMAP treatment were first observed at 22 months of age, by which time 45% of the individuals had already formed a brown-yellow tissue that fully or partly covered the conical appendages. The rest of them appeared to have no gametogenic development (Plate 2A,C). The surface of the brown-yellow gonads was smooth. These characteristics remained until the end of the study, no diploid-like gonad development being observed. Percentages of individuals with the brown-yellow gonads appeared consistent over time, i.e., 42 and 43% at 28 and 31 months of age respectively. Further histological examination confirmed that the brown-yellow gonads were male and the undeveloped ones female (see Section Histological examination of gametogenesis). A χ^2 test did not reject ($P > 0.05$) an equal sex ratio of 1:1 for triploid abalone (pooled data for 28 and 31 months of age), nor for control diploid abalone. Gonad area and gonad area index of male triploid abalone followed a very similar trend to that of diploid abalone over time (Figures 9 and 10). There were no differences in either variable between triploid (male only) and diploid abalone at the end of the study. It should be noted that variability among replicate raceways was not included in the analysis. This imposes a risk of increasing Type I error (Underwood 1997). A result of no differences between treatments of the test is however valid because it cannot lead to an excessive Type I error.

Abalone in the CB treatment displayed gonads that either resembled those of control diploids or those of 6-DMAP triploids. It was therefore possible to separate triploids and diploids putatively in CB treatment (during spawning seasons); that is, those individuals with a reddish or cream-white gonad were identified as diploid and those with brown-yellow or no gonads, as triploid.

Histological examination of gametogenesis

Ault (1985) classified ovarian development of diploid abalone into five phases (pre-spawning histology): (1) pre-proliferative phase where the gonad is essentially immature and little or no germinal epithelium is evident; (2) proliferative phase where squamous germinal epithelial cells growing from trabeculae become oogonia ranging from 10 – 25 μm in size and forming clusters on the walls of the trabeculae; (3) new stalk phase where oogonia become stalked at about 25 μm and are grouped in clusters. At this stage these cells termed primary oocytes and vitellogenesis initiates and (4) old stalk phase where primary oocytes are > 50 μm in diameter and vitellogenesis is intensive. The oocytes each remain attached by a stalk and appear tear-shaped; and (5) free phase where upon reaching a diameter of 170 – 190 μm, oocytes detach from trabeculae and each is completely surrounded by a chorion. Free oocytes are present in the inter-trabecular lumina and in the major lumen between the digestive gland and lumina.

Gonads of diploid abalone were mostly immature until 22 months of age (sex was indeterminate), despite few of them having advanced to the new stalk phase. At 25 months of age however, ovaries of diploid abalone all reached free phase (Plate 3E) and large amounts of spermatozoa filled the inter-trabecular lumina (Plate 3F) in males. This uni-developmental stage of each sex did not change perceptibly until 31 months of age, when post-spawning histology was observed in some specimens. Of the ten specimens examined, one female was void of free oocytes (new and old stalk phase) (Plate 4A) and another showed shrunken oocytes in several lumina, indicating reabsorption (Plate 4B). It was virtually impossible to discern whether a male had spawned unless it had done so completely. An increased number (area) of trabeculae with reduced amounts of spermatozoa in the lumina (Plate 4C) at this age did however suggest that males had indeed partially spawned.

Active gonadal development of triploid abalone in the 6-DMAP treatment was absent prior to 19 months of age with no or little germinal epithelium present. At 22 months of age, a thin layer of germinal cells was apparent in all specimens examined, regardless of whether or not a brown-yellow gonad was present at the macroscopic level (see Section Visual examination of gonads). But it was still difficult to sex the animals at the microscopic level. At 25 months of age, plentiful spermatocytes were detected in all brown-yellow gonads (Plate 3B,D), and a thin layer (200 – 250 μm thick on the sections throughout the study) of oogonia (< 25 μm in diameter) in those lacking an apparent gonad development macroscopically (Plate 3A,C). Gametogenesis was arrested at this developmental stage in each sex through to the end of the study. No spermatids, spermatozoa and post-vitellogenic oocytes were detected.

Gametogenesis of the putative triploid and diploid abalone in the CB treatment was identical to that of 6-DMAP triploid and control diploid abalone, respectively, when examined at 28 months of age.

4.1.5.5. *Discussion*

Due to time constraints on this report, results discussed are limited to abalone up to 31 months of age.

Growth

Up to 31 months of age triploid blacklip abalone (*H. rubra*) had not grown faster than sibling diploids. Lack of detectable differences may be because the recently matured diploid abalone had not yet become fully sexually active, as indicated by the concurrent occurrence of growth and gonadal maturation at this time (Figures 2, 3, 9 and 10). In the Sydney rock oysters *S. commercialis*, a growth advantage of triploids did not become apparent until the second summer spawning season (Nell *et al.* 1994; Hand *et al.* 1998). Similarly in the clam *M. mercenaria*, triploids surpassed diploids only after diploids had experienced at least two spawning periods (Eversole *et al.* 1996).

It is therefore likely that triploid *H. rubra* will eventually reach a larger body size. To have commercially useful benefits, this would need to occur prior to the attainment of optimal marketable size of 80 mm shell-length that would be reached within in a further period of six months of culture on extrapolated growth data obtained in this study. On the other hand, as abalone were fed only on an artificial diet and as food variety (quality) is one of the important factors affecting growth and/or reproduction in molluscs (Fleming *et al.* 1996; Navarro *et al.* 2000, 2002), the relative performance of triploid to diploid abalone may vary with the diet used. Nevertheless, it is highly relevant to evaluate performance of triploidy based on the use of artificial diet, which is the common practice for commercial culture of *H. rubra* in Australia. Results of this study are consistent with those of Nell *et al.* (1994), Eversole *et al.* (1996), Hand *et al.* (1998) and Ruiz-Verdugo *et al.* (2000) in that growth of triploid molluscs is not much different from their diploid counterparts prior to sexual maturation or active spawning.

Chemical induction of triploidy as used in this study generated a mixture of triploids and diploids (within-batch diploids). It appears that some researchers have taken advantage of these within-batch diploids by using them, instead of the untreated diploids, as controls (Stanley *et al.* 1984; Tabarini 1984). Results based on such practices are biased because performance of the within-batch diploids can be very different from that of the untreated diploid controls (Ruiz-Verdugo *et al.* 2000; Maldonado-Amparo *et al.* 2004). In the present study, the within-batch diploids (CB-2N) in CB treatment were in fact found to have the largest body size of all at 28 and 31 months of age. Although the underlying causes are not known, inadvertent selection pressure imposed by toxic chemicals used to induce triploidy could probably have played an important role in the observed difference (the more vigorous diploid individuals surviving treatment for whatever reason). The discrepancy in body size (between CB-2N and CB-3N) can actually be tracked back as early as 22 weeks of age. A small difference at the start may result in considerable differences later on, as can be demonstrated by the grading effect (Krsinich, personal communication 2004).

It is also possible that presence of the larger CB-2N individuals suppresses the performance of CB-3N individuals as mediated by behavioural interaction between the two ploidy types. In fish, triploids are found to be less aggressive than diploids (Benfey 1999). It is therefore more pertinent to test the overall effect of CB treatment (combined data of CB-2N and CB-3N) in the present study.

Heasman *et al.* (2004) reported a length-age relationship of SL (μm) = $0.015X^2 + 49.643X - 982.16$ (where X = days post fertilisation) for *H. rubra* reared at the same research unit based on growth data of 13 batches of juveniles. Abalone in the present study reached approximately a mean shell length of 25 and 60 mm at 16 and 31 months of age respectively, which were close to that of 26 and 58 mm at the corresponding ages as predicted by this equation. Therefore, there is no indication that animals used in the present study had under-performed due to suboptimal conditions. The overall annual growth rate of 25 mm shell length for *H. rubra* achieved in the present study was higher than that of about 18 mm for *H. rubra* in a 5-month study (Huchette *et al.* 2003) and of 16 mm for the greenlip abalone *H. laevigata* in a 2.5-year study (Reaburn and Edwards 2003) under commercial conditions, in southern Australia. Intra-specific difference in growth may be attributable to genetic variation resulting from origin of stock or may simply reflect varying culture conditions including husbandry. The warmer year-round ambient sea temperatures at the site of the present study, which is close to the northern limit of the geographical range of *H. rubra*, may also favour faster growth of this species in the central or the north coast of NSW than at more southern areas of the range (Heasman *et al.* 2004).

Shell growth of *H. rubra* was slightly curvilinear (Figure 2), with no suggestion of seasonal variation (Preece and Mladevov 1999). In accordance with this growth, body weight increased at an ever-increasing rate over time (Figure 3). The practical implication is that adherence to optimal performance of abalone in the final year of culture is the most crucial in determining the overall market turn off period and hence economic viability of farming.

The weight-length relationships seemed to be akin for abalone in the two triploidy treatments and diploid control. But slopes of the regression lines fitted to log transformed data were found significantly different from each other. The weight-length relationships obtained in the present study is similar to that of BW = $0.0001 \times SL^{3.146}$ for (diploid) *H. rubra* previously cultured at the same research unit (Heasman *et al.* 2004).

Scant attention has been paid to the effects of triploidy on shell morphology in molluscs. Tabarini (1984) reported greater shell inflation for triploids scallops *A. irradians*, but Manson *et al.* (1988) report the opposite for the clam *A. arenaria*. Here, triploid *H. rubra* were found to have a more elongate shell than sibling diploids. Although narrower shells were related to faster growth in wild

(diploid) populations of *H. rubra* (Worthington *et al.* 1994), this was not found to apply to the narrow shelled triploid *H. rubra* in the present study.

No difference in survival was found between triploid and diploid abalone in the present study. This result is consistent with that of Barber and Mann (1991) and Nell *et al.* (1994), on the American oyster *C. virginica* and the Sydney rock oyster *S. commercialis* respectively. However, subsequent research by Matthiessen and Davis (1992) and Hand *et al.* (1998) found significantly greater survival for triploids compared to diploids of the same two species.

Percent triploidy was stable in the 6-DMAP treatment but it was not in the CB treatment. Since the overall mortality throughout the study was low, within treatment differential mortality of triploids and diploids, if any, does not seem to explain the decreased levels of triploidy over time in the CB treatment. As mortality was not monitored during the nursery phase, it is not known whether the decrease in triploid percentage in CB treatment (from 22 weeks to 19 months of age) had already happened (due to differential mortality of triploids and diploids within treatment) before the experiment was established. However, triploid/diploid mosaics and reversion of triploids to diploids (Allen *et al.* 1996; Hand *et al.* 1999) cannot be ruled out, nor can whether the mosaics and reversion are method of triploidy induction dependent.

Reproduction

A reduction in gonad size is often found to be associated with the sterility of triploid molluscs (Allen *et al.* 1986; Komaru and Wada 1989; Cox *et al.* 1996). However, triploids of the scallop *A. irradians* bear a heavier gonad than diploids (Tabarini 1984). In the present study, female triploid *H. rubra* lacked an apparent gonad at the macroscopical level, but males developed a gonad (filled with germinal cells but not connective tissues, Plate 3B) of similar size to diploids despite retarded gametogenesis. Therefore with respect to energy utilisation, cost of initial gonad development is still much of a burden on somatic growth for male triploid *H. rubra*. In fact the elevated foot muscle indices of 6-DMAP induced triploid *H. rubra* (Figure 7) occurred in females rather than males (data not shown). Since it is the size of the foot muscle that determines a price in the market, it then seems that triploidy may have some merit in *H. rubra*, but probably limited to females only. Nevertheless, the higher foot muscle indices of triploid abalone need to be further confirmed, because foot flesh component of mature diploid abalone showed substantial seasonal variation, in association with the storage and the subsequent use of energy for reproduction (Hayashi 1983).

Under the captive rearing conditions provided in the study, gonadal maturation was not followed by a mass spawning across the diploid populations. This was possibly due to a lack of environmental cues or a lack of spawning opportunity due in turn to the fact that minimum requirements for full development of gonad, i.e., 120 – 240 days at favourable temperatures of 15° – 18C (Savva *et al.* 2000; Grubert and Ritar 2003a,b) were not satisfied. In the present study, first attainment of sexual maturity only occurred of the final six months of culture (25 – 31 months of age). The subsequent effect of energy redistribution (from gonad to somatic tissue due to reabsorption of gametes following spawning seasons) on growth of diploid abalone, and thus the relative performance of triploids and diploids, therefore needs to be further addressed. Moreover, in more protracted studies, even if all diploid *H. rubra* had sufficient time to fully develop their gonads and spawn, the significant loss in overall soft tissue weight may have been masked by the large relative size of the foot muscle, which accounts for nearly 70% of the dry soft tissue weight at first sexual maturity (Figures 6 and 7). This is in contrast with bivalves such as the Pacific oysters (*C. gigas*) that expend as much as 64% of dry tissue weight during their first spawning at one year of age (Allen and Downing 1986). Accordingly, major marketable flesh yield benefits conveyed by triploidy in oysters cannot be expected to extend to farmed abalone.

Gonad discoloration associated with triploidy has only been reported for a few species of mollusc. This is characterised by distinct light to dark brown patches on the gonad surface in the oyster *S.*

commercialis (Hand *et al.* 1998; Hand and Nell 1999), or by a brownish gonad in the scallop *A. ventricosus* (Ruiz-Verdugo *et al.* 2000). In the present study, a distinctive brown-yellow gonad was observed in male triploid abalone. Regardless of the cause, a discoloured gonad may adversely affect the marketability if it is to be part of the final product. Fortunately, this is not the case with abalone.

Gametogenesis is generally retarded in triploid molluscs but to varying degrees according to species and sex. In extreme cases, ovaries in triploids typically contain oogonia coupled with sparsely distributed post-vitellogenic oocytes, while the testes in triploids contain spermatocytes with few or no spermatids/spermatozoa present (Komaru and Wada 1989; Barber and Mann 1991; Cox *et al.* 1996; Eversole *et al.* 1996). Triploid *C. gigas* and *P. martensii* are able to produce mature gametes that although being much reduced in number compared with diploids are nevertheless viable or fertilisable (Guo and Allen 1994a; He *et al.* 2000). In triploids of the hermaphrodite scallops *A. ventricosus* (Ruiz-Verdugo *et al.* 2000) and *Nodipecten subnodosus* (Maldonado-Amparo *et al.* 2004), gametogenesis of both ovarian and testicular portions is also greatly retarded and in the former species, the testicular portion of gonad was gradually replaced by the female one with progressive age.

Results of the present study show that gametogenesis is severely retarded in triploid *H. rubra*. The assumption by some authors such as Beaumont and Fairbrother (1991) that triploidy confers sterility was based on the likely inability of three chromosome sets to properly synapse during meiosis. A significant connotation of such assumption is that offspring of triploid molluscs are unlikely to be viable (Stöck *et al.* 2002). Triploidy on its own, does not prevent reproductive activities such as gametogenesis, vitellogenesis, gamete maturation and even spawning, which are under the tight control of sex steroids or hormones. Tiwary *et al.* (2002) suggests that in triploid fish the low activity of the neurosecretory cells (responsible for the secretion of gonadotropin-releasing hormone) may be due to the lack of positive feedback stimulation by sex steroids and/or reduced responsiveness of sensory cells to environmental cues required for gonadal maturation. Through appropriate hormone stimulation, triploid fish are able to synthesise and secrete physiological levels of vitellogenin (Benfey *et al.* 1989; Benfey 1999). Unfortunately the equivalent neuroendocrine mechanisms controlling reproduction in diploid, and therefore triploid molluscs, remains poorly understood.

The sex ratio of triploid *H. rubra* did not deviate from 1:1. Likewise, triploidy status did not affect sex ratio in *C. gigas* (Allen *et al.* 1986) and *S. commercialis* (Cox *et al.* 1996), but hermaphroditic individuals were observed for both species. In contrast, Guo and Allen (1994b) found significantly higher numbers of males in triploid clams *M. lateralis*.

In conclusion, juvenile triploid and diploid *H. rubra* were very similar in the many traits evaluated apart from a significant difference in shell shape during the first 31 months of life. Gonadal development and gamete maturation were abnormal in triploid abalone compared to sibling diploids. While female triploids lacked an apparent gonad at the macroscopical level, microscopic examination revealed a layer of oogonia. In contrast, male triploids were able to form a gonad of similar size as diploids albeit with brown-yellow discolouration and halted gametogenesis at spermatocyte formation. Similar results were obtained at the time of writing (34 months of age when abalone reached a mean shell length of about 66 mm).

4.1.5.6. References

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Table 1.   Body size (means ± SE) of triploid and diploid *H. rubra* at the start and the end of
a two-year study.

Treatments	Initial (*n* = 60)		Final (*n* = 40)	
	SL (mm)	**BW (g)**	**SL (mm)**	**BW (g)**
6-DMAP	9.1 ± 0.2	0.14 ± 0.01	59.1 ± 0.7	35.3 ± 1.2
CB	9.4 ± 0.3	0.15 ± 0.01	60.9 ± 0.9	36.4 ± 1.4
CB-3N			*57.8 ± 1.2 (n = 18)*	32.1 ± 2.0 (*n* = 18)
CB-2N			*62.8 ± 1.1 (n = 22)*	39.1 ± 1.8 (*n* = 22)
2N	8.1 ± 0.2	0.10 ± 0.01	59.2 ± 0.7	34.0 ± 1.1

SL: shell length. BW: body weight. 6-DMAP: triploids produced with 6-DMAP. CB: triploids produced with
CB, containing a mixture of triploids (CB-3N) and diploids (CB-2N). 2N: diploids.
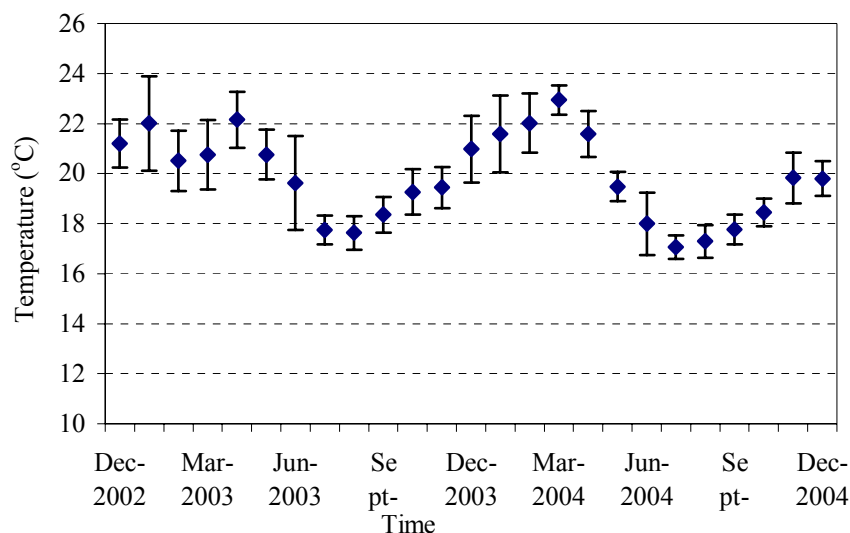
Figure 1. Mean monthly seawater temperatures of the study site over a two-year study (December 2002 to December 2004) (means ± SD).
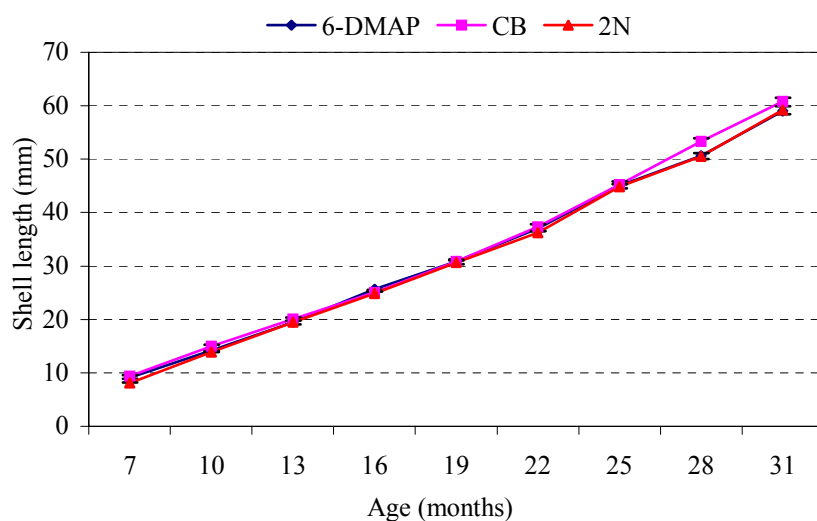
Figure 2. Mean shell length (means ± SE, *n* = 40 – 75) of triploid and diploid *H. rubra* over a two-year study. 6-DMAP: triploids produced with 6-DMAP. CB: triploids produced with CB (containing a mixture of both triploids and diploids). 2N: diploids.
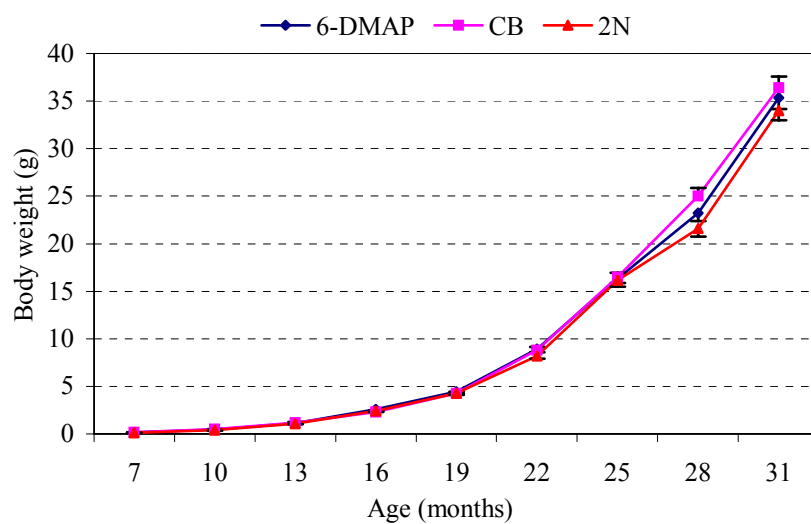
Figure 3.   Mean body weight (means ± SE, n = 40 – 75) of triploid and diploid *H. rubra* over a two-year study. 6-DMAP: triploids produced with 6-DMAP. CB: triploids produced with CB (containing a mixture of both triploids and diploids). 2N: diploids.
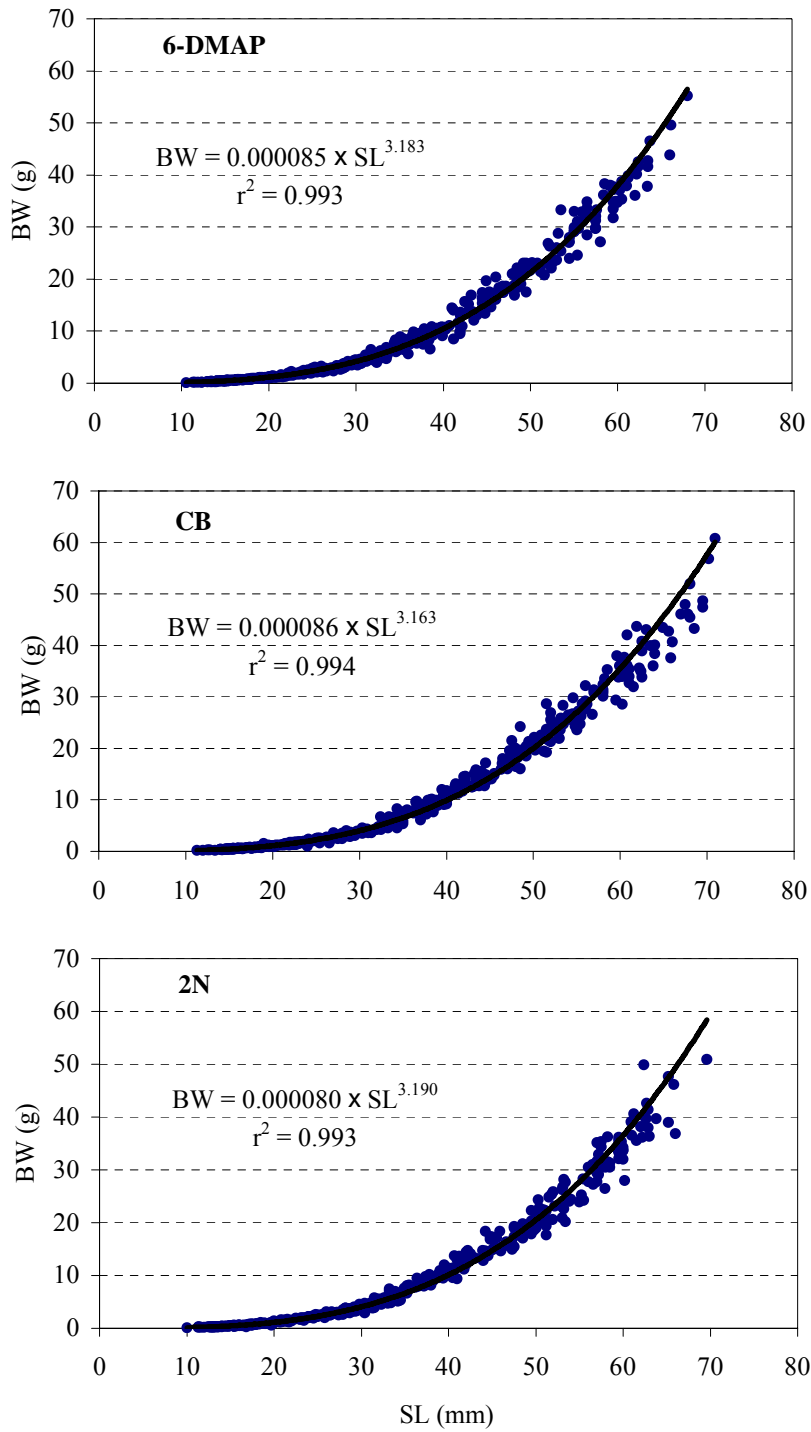
Figure 4. Shell length (SL) and body weight (BW) relationships of triploid and diploid *H. rubra*. 6-DMAP: triploids produced with 6-DMAP. CB: triploids produced with CB (containing a mixture of both triploids and diploids). 2N: diploids.
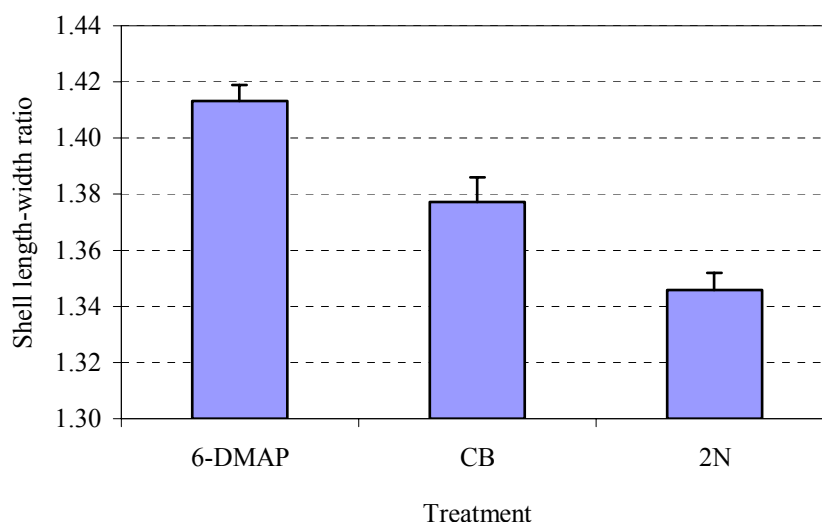
Figure 5. Shell length-width ratios (means ± SE, *n* = 40) of triploid and diploid *H. rubra* at 31 months of age. 6-DMAP: triploids produced with 6-DMAP. CB: triploids produced with CB (containing a mixture of both triploids and diploids). 2N: diploids. All means are significantly different (*P* < 0.01) from each other.
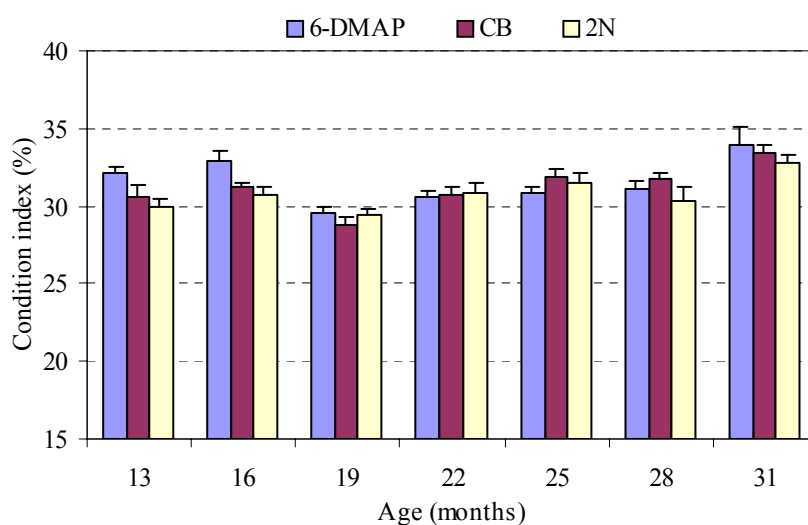
Figure 6. Condition indices (raceway means ± SE, *n* = 5) of triploid and diploid *H. rubra* over time. 6-DMAP: triploids produced with 6-DMAP. CB: triploids produced with CB (containing a mixture of both triploids and diploids). 2N: diploids.
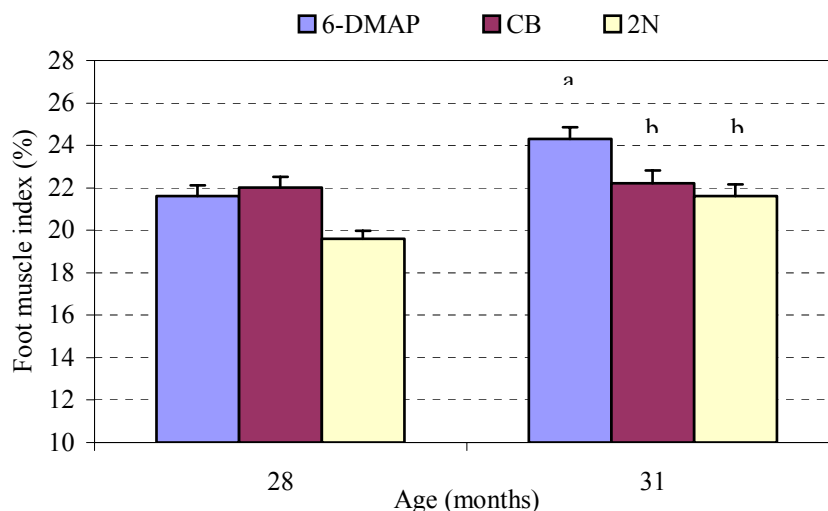
Figure 7. Foot muscle index value (means ± SE, *n* = 20 – 25) of triploid and diploid *H. rubra* after diploids had reached sexual maturation. 6-DMAP: triploids produced with 6-DMAP. CB: triploids produced with CB (containing a mixture of both triploids and diploids). 2N: diploids. Bars sharing different superscripts differ significantly (*P* < 0.05).
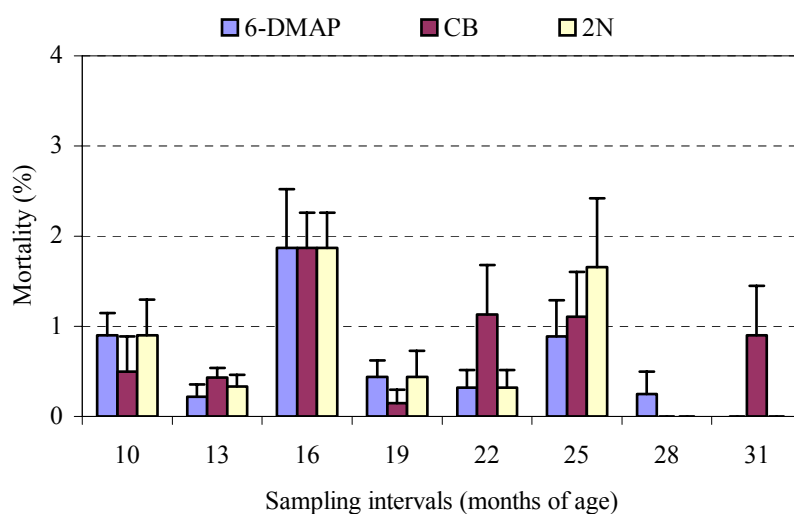
Figure 8. Mortality (means ± SE, *n* = 5) of triploid and diploid *H. rubra* over a two-year study. 6-DMAP: triploids produced with 6-DMAP. CB: triploids produced with CB (containing a mixture of both triploids and diploids). 2N: diploids. Sampling intervals: each represents a three-month interval from the age of previous sampling.
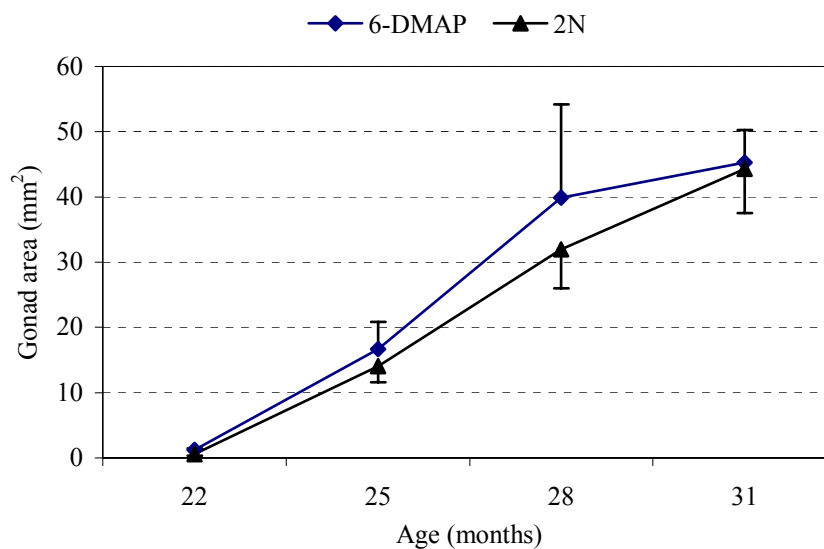
Figure 9. Gonad area (means ± SE) of triploid (*n* = 4 – 6) and diploid (*n* = 10) *H. rubra* over time. 6-DMAP: triploids produced with 6-DMAP (containing males only). 2N: diploids.
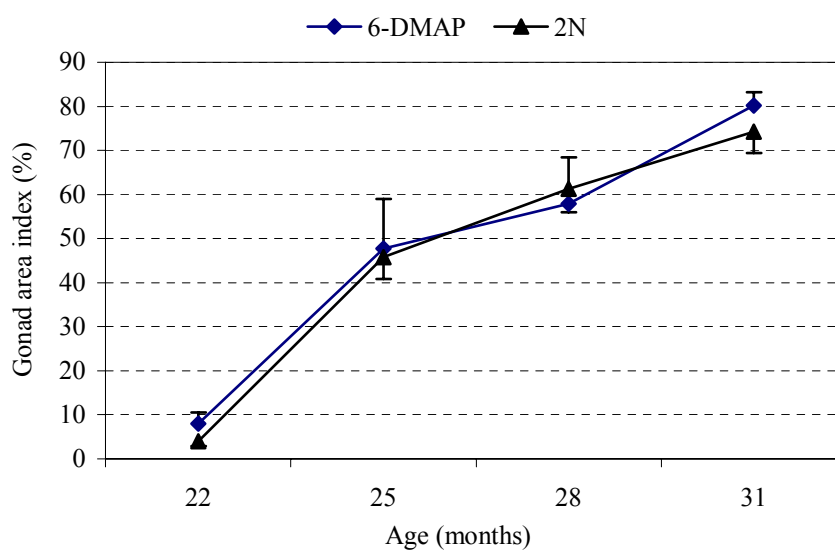
Figure 10. Gonad area index (means ± SE) of triploid (*n* = 4 – 6) and diploid (*n* = 10) *H. rubra* over time. 6-DMAP: triploids produced with 6-DMAP (containing males only). 2N: diploids.

Plate 1. A layout of the larger culture raceways (side by side = 60 × 40 cm).
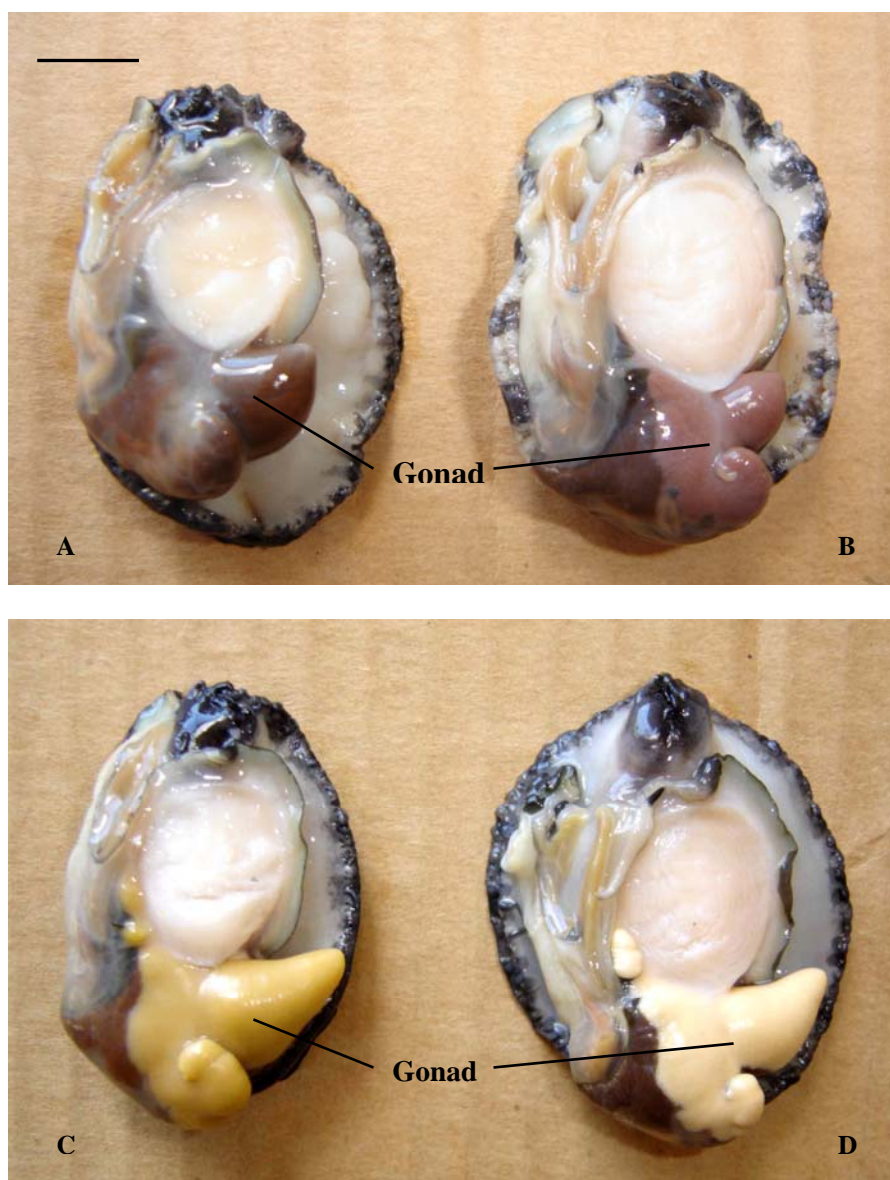
Plate 2.   Gonads of triploid (produced with 6-DMAP) and diploid *H. rubra* at 28 months of age. A: a conical appendage of female triploid abalone that had no obvious gametogenic activity. B: a reddish gonad of female diploid abalone. C: a brown-yellow gonad of male triploid abalone. D: a cream-white gonad of male diploid abalone. Bar = 10 mm.
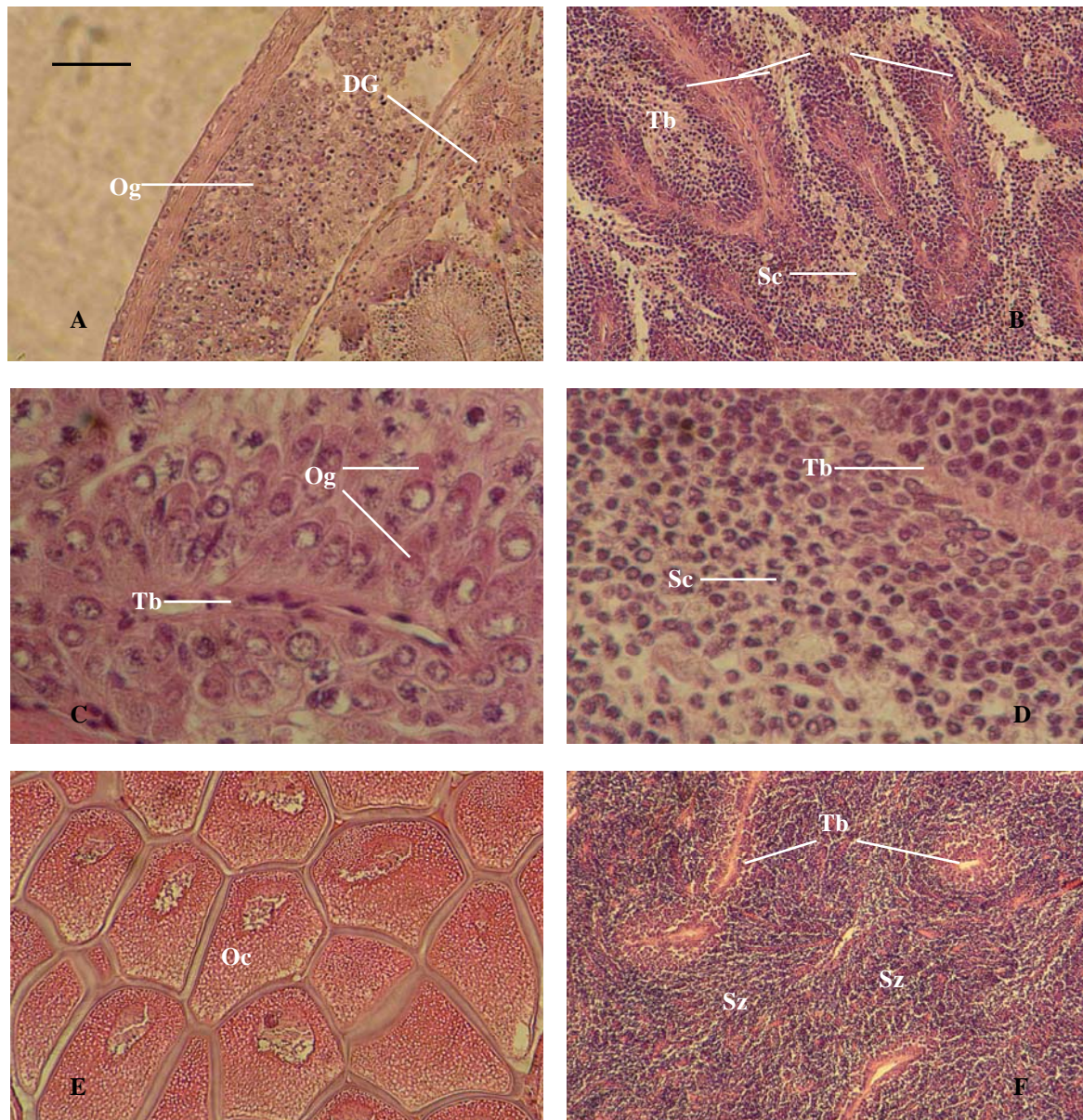
Plate 3. Gametogenesis of triploid (produced with 6-DMAP) and diploid *H. rubra* at 28 months of age. A, C: female triploids only developed a thin layer of oogonia. B, D: male triploids underwent extensive gametogenesis but further development was arrested at formation of spermatocytes. E: female diploids with ripe oocytes. F: male diploids showing large areas of dense spermatozoa in the inter-trabecular lumen. DG: digestive gland. Oc: oocyte. Og: oogonia. Sc: spermatocyte. Sz: spermatozoa. Tb: trabecula. Bar = 100 μm for A, B, E and F; and = 25 μm for C and D.
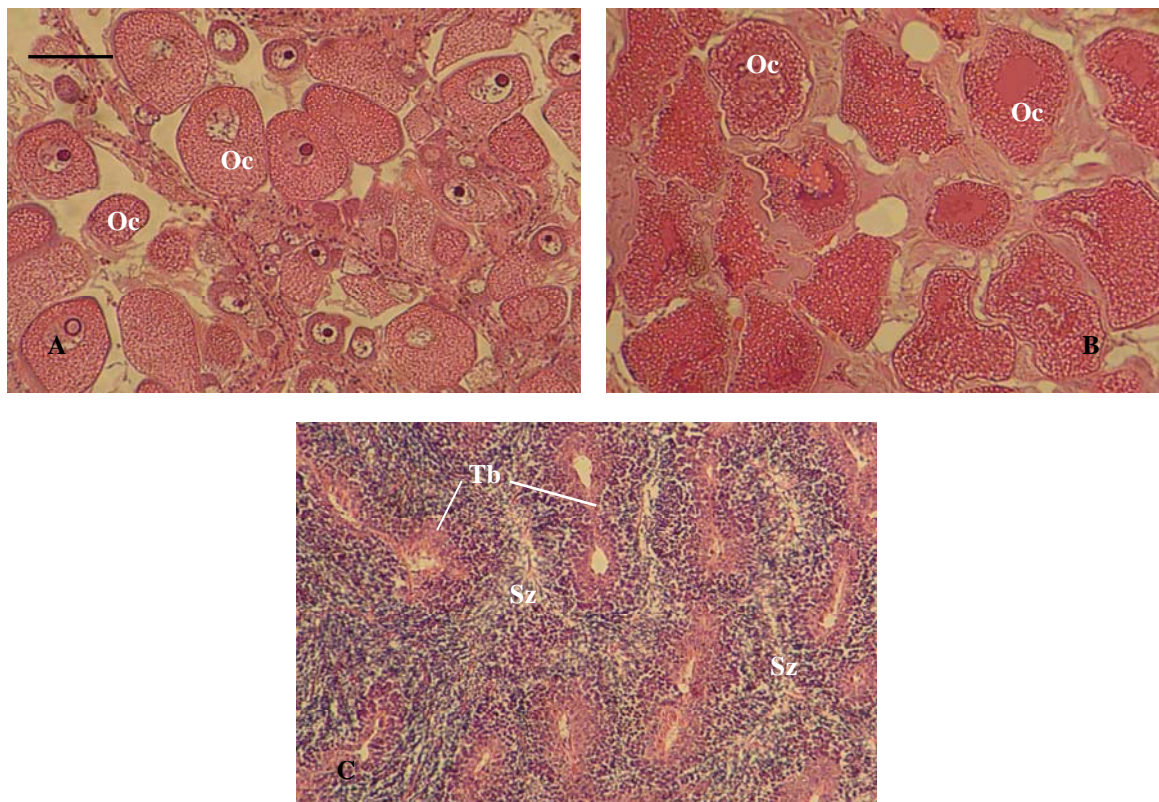
Plate 4.   Post-spawning histology of gonads in diploid *H. rubra* at 31 months of age. A: an ovary that had primary oocytes (new and old stalk phases) but lacked ripe ones. B: an ovary showing shrunken oocytes being reabsorbed. C: a testis with increased numbers (areas) of trabeculae but fewer spermatozoa present in between (compared to Plate 3F). Oc: oocyte. Sz: spermatozoa. Tb: trabecula. Bar = 100 μm.

4.2. Evaluation of factors affecting post-release survival of hatchery reared juveniles

Two complimentary field experimental programs were used to address mortality of cultured juvenile abalone seeded to natural reef habitat. The first, reported in full below (Section 4.2.1), comprised assessment of several factors on early post-release survival using a series of experiments in which small juveniles were seeded in small numbers at moderately density over small spatial scales to natural habitat.

The second program, described below (Section 4.2.2), culminated in a single experiment conducted on a much larger spatial scale. A key objective of this experiment that was commenced in July 2005, is to assess the merits, or otherwise, of seeding habitat depleted of wild stocks, at very low densities typical of healthy self-sustaining populations (Prince *et al.* 1988; McShane 1991; Shepherd *et al.* 2000). Unfortunately accurate assessment of the survival of stock released in this manner is not practically achievable for 3 to 4 years. Accordingly, results of this ongoing experiment that is also assessing long term effects of seeding large numbers of cultured seed on gene pools of depleted wild populations will be separately published at a later date.

4.2.1.   Evaluation of factors affecting post-release survival of hatchery reared juveniles: Moderate density

Experimental assessment of the survival of cultured abalone (*Haliotis rubra*) seeded in small numbers to natural habitat at Port Stephens, NSW

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4.2.1.1.   Abstract

Releasing juvenile abalone reared in aquaculture into natural habitats has the potential to enhance and/or re-establish wild populations of abalone, and has been trialled in many countries with limited and variable success. There is, therefore, strong impetus for an experimental approach to better understand which factors are important to the survival of cultured *H. rubra* seeded to the wild. A substantial amount of evidence suggests that initial mortality of abalone is density-dependant and it is now acknowledged that seeding juveniles above their natural densities may be a major reason for the failure of reseeding programs. A major aim of the current study therefore, was to assess survival of hatchery-reared *H. rubra* when seeded in small numbers. The target of any seeding program is to place juveniles directly into natural habitat whilst maximising protection from predation and minimising physiological stress. To this end, we developed a cheaply and easily-made seeding device into which abalone can be seeded and transported with minimal stress. These devices can be wedged easily and quickly into suitable microhabitats and remain secure there, allowing *H. rubra* access to their natural habitat and protection from predation whilst they acclimatise to their new surroundings.

The recent Environmental Impact Statement (EIS) for the NSW Abalone Fishery (2005) raised the need for experimental investigations done at small-scales to better understand the potential of reseeding attempts in NSW. In the current study, therefore, button-size *H. rubra* were seeded in moderate densities ($100 - 120/m^2$ per1 m^2 site) in a series of small-scale experiments to test: (i) the importance of the spatial and temporal configuration at which abalone are seeded, (ii) the usefulness of using the sea urchin, *Centrostephanus rodgersii* to act as a natural defence and potential shelter from predators, and (iii) whether the diet on which the animals were raised affects the survival of *H. rubra* once in the wild. All experiments were done at Port Stephens and were sampled at daily, weekly, fortnightly, monthly and $2 - 3$ monthly intervals until the completion of the project (approximate total of 6 months for each experiment).Within days, total recovery among treatments in any experiment ranged between 7.6 % – 30.6%. The spatial configuration at which abalone were deployed and their proximity to sea urchins had little effect on their long-term survival. Total recovery among treatments dropped to a consistent 0.6 % – 1.8% from $1 - 2$ months, with recovery reduced further after 6 months (0.05 % – 0.6 %). Seeding fewer abalone can sustain populations of abalone close to the natural densities of adults which have been observed in previous studies in NSW ($1 - 3/m^2$). This may mean that it is cost-effective to seed fewer, larger abalone, which are known to survive better than small abalone.

4.2.1.2.   Introduction

Blacklip abalone (*Haliotis rubra* Leach) are the major commercial species of abalone endemic to south eastern Australia. They are a small but valuable fishery for NSW, with over $200 million gained by exports of abalone in 2003 (NSW Fisheries). Almost 5000 t of *H. rubra* are taken each

year in Australia, a haul that represents about 40% of the total world catch (McShane 1999), making blacklip abalone a species of considerable importance to commercial fisheries of the world. Many of the world's abalone fisheries have collapsed, but Australia's fishery remains a highly valuable industry of economic importance. Nevertheless, the sedentary nature of abalone, their tendency to aggregate and the high dollar value (close to AUS $100 kg $^{-1}$) on Asian markets make abalone particularly susceptible to overfishing (Breen 1992). The abalone fishery in NSW is mainly based from populations of *H. rubra* in the southern region of the state, and annual catch rates have declined by almost half from the 1970s to the 1990s (McShane 1999).

Natural re-colonisation of depleted populations of abalone is limited, because larval abundance and frequency may be insufficient to sustain adult populations (McShane 1995a), or because dispersal of larvae is too localised to flow among neighbouring populations (Prince *et al.* 1988, McShane *et al.* 1988, McShane and Smith 1991). Second, low fertilization success, the transport of larvae away from suitable benthic habitat (e.g., boulders, crevices, algae) and high predation of larvae (Shepherd *et al.* 1992) are all likely to contribute to low rates of natural re-colonisation of localised populations. If fishing continues and where it is continued, the decline of natural populations and concomitant loss of economic generation for NSW and Australia would be substantial. Furthermore, the impact on the ecology of rocky reefs by severely reduced populations of abalone is unknown.

Following the success of year-round seed production and rearing of juveniles in aquaculture in Australia (Heasman *et al.* 2004), seeding small, hatchery-reared juveniles into natural habitat may be a viable, year-round and efficient method to enhance and/or re-establish populations of abalone. The concept of mass planting of cultured abalone to enhance natural stocks was pioneered by the Japanese in the 1960s and subsequent attempts have been trialled for many species of abalone in California (Tegner and Butler 1985, 1989, Davis 1995, Ebert and Ebert 1988, Rogers-Bennett and Pearse 1998, McCormick *et al.* 1994, Lapota *et al.* 2000), South Africa (de Waal and Cook 2001, de Waal *et al.* 2003), Australia (Preece *et al.* 1997, review in Shepherd *et al.* 2000) and New Zealand (Tong *et al.* 1987, Schiel 1992, 1993). Unfortunately, these attempts are typically fraught with difficulty. The particular characteristics of abalone (e.g., life-history, habitat-requirements and ecological interactions) may make Haliotids particularly difficult to re-seed compared to other molluscs (e.g., bivalves) (Hilborn 1998).

The survival of abalone seed depends on many factors, including the condition and size of seed (Kojima 1981, McCormick *et al.* 1994), the habitat into which seed is planted (Schiel 1993), the availability of food and shelter from predation (Shepherd and Turner 1985, Shepherd *et al.* 2000) and the planting method: e.g., hand planting (Tegner and Butler 1985) or the use of intermediate habitat (Ebert and Ebert 1988). It is likely that these factors are spatially and temporally variable (e.g., Poore 1972, Schiel 1993) and have varying degrees of importance for different species in different habitats of the world. Moreover, it is highly likely that these factors interact rather than act in isolation to affect the survival of juvenile seed, thus further complicating the success of re-planting strategies. The broad aim of any reseeding attempt is to ensure juveniles are placed directly into suitable habitat whilst maximising protection from predation and minimising physiological stress. There is much need, however, for research to understand the processes that affect survival of seed in the wild, so we can better valuate reseeding procedures.

Stresses associated with travel and handling can cause substantially reduced survival of juvenile abalone in the wild. Poor physical condition and thermal shock can lead to high rates of instantaneous mortality (McCormick *et al.* 1994). Stressed abalone may also be more susceptible to predation because, when stressed, they often produce mucus which attracts predators (Tegner and Butler 1985). Many authors advocate using natural (e.g., oyster shell) or artificial substrata to seed abalone, thereby substantially reducing physiological stress (Ebert and Ebert 1988, Tegner and Butler 1989, McCormick *et al.* 1994). Abalone can be seeded to these substrata in the hatchery,

easily transported as a unit (with adequate cooling and moisture) and placed directly into the natural habitat with minimal planting effort and stress to the seed. Ideally, such planting devices should be designed to provide some initial protection from predators. In Australia and New Zealand, a variety of methods to deploy abalone larvae have been developed (Tong *et al.* 1987, Schiel 1992, Preece *et al.* 1997), but devices to deploy juveniles, such as boulders covered with encrusting coralline algae and large, rectangular PVC containers, have met with limited success because abalone were crushed, preyed upon or the device was unstable in the natural habitat (Heasman *et al.* 2004). As part of the current study, we developed device into which abalone can be seeded and transported with minimal stress and that could be wedged securely into suitable microhabitats and provide protection from predation.

Pioneering work in Japan has shown much greater levels of survival of abalone seed when they are larger at the time of planting (Inoue 1976, cited in Tegner and Butler 1989). Clearly, there is a trade-off between the cost of rearing abalone to large sizes in aquaculture, which is often prohibitive, and their survival in the wild. Because many studies wish to seed large numbers of juvenile abalone, they are often constrained to using small "button-size" juveniles (< 20 mm) (Tong and Moss 1992). Larger sizes (up to 70 mm) have been seeded in California and Japan, but button-size is still currently the most economical for Australia because growing any larger in the hatchery is limited by space and the cost of food (Heasman *et al.* 2004).

Predation is a substantial cause of high mortality of seeded abalone (Shepherd *et al.* 2000). Juvenile abalone (10 – 30 mm) are mainly preyed upon by whelks, crabs, octopus, wrasses and morwongs, which often preferentially forage in the habitat of juvenile abalone (Shepherd and Turner 1985). Introducing large numbers of potential prey at one time may attract significantly more predation than would occur at natural densities, and opportunistic predators and scavengers may be attracted to release sites (Tegner and Butler 1989). As an example, blue-throated wrasses (*Notolabrus tetricus*) are an important predator of juvenile *Haliotis laevigata* (greenlip abalone) in South Australia and were shown to increase consumption with increasing density of abalone (Shepherd and Clarkson 2001). Hatchery-reared abalone may be particularly susceptible to predation once seeded, as they have been observed to take longer to exhibit behaviour associated with avoidance of predators: hiding in cracks and crevices (Schiel and Weldon 1987). This remains untested in the wild and there are reports from field studies that abalone were quick to move toward crevices (Tegner and Butler 1989). Nonetheless, excluding predators from release areas using enclosures has significantly increased survival of juveniles (Kojima 1981). As debate continues about whether seeded abalone are more vulnerable to predation and are preyed upon to a greater extent once seeded, it seems prudent to include some form of protection from predators whilst abalone acclimatise to their habitat.

The type of habitat into which abalone are seeded has also been shown to play an important role in their survival, particularly in areas where predation is minimal (e.g., Schiel 1993). In particular, the features of the substratum or microhabitats must provide abalone with adequate shelter and food (Kojima 1981). Different species of abalone can have different habitat requirements and juveniles and adults are often found in distinctly different habitats. *Haliotis rubra* settle to the interstitial spaces between boulders in rocky reef habitats and show very little movement out of this microhabitat in the first stages of growth (up to 6 months) (Shepherd 1986). In this microhabitat, they are protected from predation and feed on coralline algae that encrust the rock surface. As adults (> 100 mm), *H. rubra* are more associated with crevices on vertical rock walls (Shepherd 1973, 1986), sheltering in these habitats during the day and emerging at night to forage on algae (Shepherd 1973, Shepherd and Turner 1985). Survival of small abalone is often increased when the habitat is chosen carefully to contain cobbles and boulders (e.g., Schiel 1993) and survival of larger abalone is increased when seeded in habitat with cracks and crevices (Shepherd 1986). Many seeding attempts have failed simply by lack of attention to the specific temperature, depth, crevice and food availability required by abalone.

Ecological interactions with other organisms, not necessarily predators within the natural habitat, may also have substantial effect on the survival of released abalone, in positive or negative ways. Sea urchins are typically abundant organisms in subtidal, barren rocky-reefs (Jones and Andrew 1990). Field and laboratory studies have shown a strong association between juvenile abalone and sea urchins (Tarr *et al.* 1996, Mayfield and Branch 2000). Small abalone are protected from predators by the sharp extended spines of the sea urchin and have a constant and localised supply of fragments of drift algae which are left over from sea urchin foraging (Tegner and Butler 1989). This seems to be a global phenomenon. In California, Rogers-Bennett and Pearse (1998) seeded *Haliotis rufescens* into sites with and without sea urchins (*Stronglyocentrotus* spp.) and later found significantly greater numbers of abalone in sites with sea urchins. Kojima (1981) found that Japanese *Haliotis discus* released in tide pools were most often found in refuge under the spines of sea urchins. In south eastern Australia, the most common sea urchin is the purple sea urchin, *Centrostephanus rodgersii*. Whilst it is negatively associated with adult *H. rubra* (Andrew and Underwood 1992), many gastropods are known to shelter under this sea urchin as juveniles, and its removal has caused significant decreases in gastropod diversity in NSW (Chapman and Underwood 2003). The presence of sea urchins in the habitat may facilitate the survival of abalone by acting as a 'natural' seeding device: offering shelter and potentially increasing the availability of food in the natural environment.

The success of reseeding programs is difficult to measure because often the numbers of seed recovered are extremely small (reviewed in Tegner and Butler 1989, Tegner 2000). Juvenile abalone are generally cryptic in crevices and underneath boulders and are difficult to see when sampling. This is further complicated by the tendency of abalone to aggregate. Such patchy distributions and the complexity of their habitat often make estimates of abalone populations using traditional quadrat and transect sampling imprecise (McShane 1995b). Shepherd (1985, 1990, Shepherd and Brown 1993) and McShane (1994, 1995b, 1998) have devoted a large number of studies to assess the best method to accurately, precisely and efficiently measure populations of abalone. Traditionally, timed swims have been used to estimate population size (Beinssen 1979) and are argued to be advantageous because any habitat unsuitable for abalone can be avoided, the method is less time-consuming and more practical and provides reliable, precise estimates (McShane 1995b, McClanahan and Muthiga 1992). There is still, however, considerable debate about the most suitable and reliable method (Gorfine *et al.* 1997, Hart and Gorfine 1997, Hart *et al.* 1997).

In addition, seeded abalone must be distinguished from native abalone within the habitat. Various methods have been tested including shell tags and notches (Tegner and Butler 1989, Shepherd *et al.* 2000), but one of the simplest methods is to manipulate the diet in aquaculture to produce a differently coloured shell (Olsen 1968). Artificial diets containing diatoms make the shell of *H. rubra* and other red-shelled abalone a bright turquoise colour which is easily identifiable in the field. Whilst this method is desirable because it is easily achieved and has been used widely, no formal tests have been done to assess whether using such a marker has potential ecological disadvantages for the seed. For example, reproduction and growth can be influenced by the amount of protein and lipids in the diet of abalone, and the contents of these elements are different in artificial vs. natural diet (Daume and Ryan 2004). Abalone reared on artificial diet may also have a reduced ability to switch from artificial diet to a natural diet of algae that is found in the wild. Moreover, abalone with bright turquoise shells may be at greater risk of predation than those with red shells because they are less camouflaged in a habitat dominated by encrusting red algae. In the current study, we include an investigation about whether the hatchery-diet of seed influenced its survival in natural habitat.

Objectives of the current study

Despite several global attempts at mass planting of abalone, there are few rigorous experimental assessments of the survival of seeded abalone (but see Schiel 1992, 1993). In Australia, there are only a handful of such studies (reviewed in Shepherd *et al.* 2000). This means that it will almost always be difficult to evaluate the success of commercial-scale out-planting, because we have inadequate knowledge about the processes causing variability in success and low rates of survival. Following a call for scientific investigations of the potential of reseeding abalone in NSW (EIS 2005), the current study is a series of small-scale experiments designed to increase our knowledge of factors that affect the survival of hatchery-reared abalone in the wild. These experiments were designed to investigate whether: (i) abalone would survive better if seeded clustered together or dispersed, (ii) seeding abalone in association with sea urchins is an efficient strategy and would maintain survival, and (iii) diet in the hatchery causes differential survival in the wild.

One of our major aims was to assess whether seeding abalone in small numbers, focusing on their placement and arrangement in the natural habitat, would result in adequate survival or matching that of natural adult densities. Recent observations have shown natural populations of *H. rubra* to be at densities of between $1 - 3/m^2$ as adults (Heasman *et al.* 2004) and $12/m^2$ as juveniles (Prince and Ford 1985), so seeding juveniles at quantities well above these numbers may be wasteful. Indeed, abalone have exhibited slower growth and less health when grown at higher densities in lab studies (Huchette *et al.* 2003). Similarly, major studies in Australia and New Zealand concur that survival of larval abalone is strongly density-dependant, even up to one year (e.g., Shepherd 1998, McShane 1991, Shepherd and Daume 1996, Preece *et al.* 1997, Shepherd *et al.* 2000). As a pertinent example, *H. rubra* showed the greatest percentage survival in Victoria at densities below $10 \ m^{-2}$, in habitat where observed densities of adults are also $3/m^2$ (McShane 1991). Increased mortality at higher densities, increased predation, and increased competition for resources could be a substantial cause of failure of programs which flood habitat with juveniles. The current study seeds small numbers of small juveniles ($100 - 120/m^2$) and supplements the shift toward strategies releasing fewer numbers ($10 - 200/m^2$), but with more care as to their protection and whereabouts (Schiel 1993, Rogers-Bennett and Pearse 1998, Hart *et al.* 1997).

4.2.1.3. Materials and methods

Blacklip abalone used in the current study were reared at the NSW Fisheries aquaculture facility at Port Stephens using parent stock from Yacaaba Head, Port Stephens (32ºS 42', 152ºE 13'). Abalone were reared to "button-size"; a mean shell length of approximately $10 - 12$ mm. Abalone were fed on an "artificial" diet of diatoms which causes a distinctive, bright turquoise colouring of the shell allowing easy identification of these juveniles *in situ*.

Development of a suitable seeding device

We designed a seeding device with the following criteria: (i) cheaply, quickly and easily made; (ii) easily placed directly into suitable microhabitat (cracks and crevices) and is stable in that habitat; (iii) abalone could be seeded to the tube with minimal handling and stress; (iv) the device would not adversely affect abalone; (iv) allow sufficient water flow and protection from predators without hindering the movement of abalone to the natural habitat.

The device (Figure 1) is based on a modified design of McCormick *et al.* (1994), made from a PVC plastic tube, 35 mm diameter and 150 mm in length. Two large circles (25 mm diameter) were drilled on alternate sides of the tube to allow the abalone access to resources in the adjacent habitat and to let water flow into the tube. Plastic mesh (25 mm) was wrapped around the tube (like a cage) to keep most large predatory fish and invertebrates out but to allow movement of juvenile abalone to adjacent habitat. One end of this cage was made into a flap so that abalone could be seeded into

the tube and the cage closed (Figure 1a). Abalone could be anaesthetised and seeded directly to the tube ready for deployment, if aquaculture facilities are near the site of release. If not, abalone can be seeded onto cut sections of sponge or other moisture-retaining material for transport (e.g., Heasman *et al.* 2004) to where they are to be released. These substrata can then be slipped inside the seeding device without stress to the abalone (Figure 1a). Alternatively, abalone can be seeded directly into the PVC tube and transported as the tubes can be packed together and kept moist and cool without crushing the abalone. A 1 kg lead weight was attached to the device to give extra stability.

The device was designed to be easily secured between boulders, and we assessed the ease at which devices could be transplanted by divers using SCUBA. We also assessed the stability of the device in natural habitats, in an experiment done at Cape Banks, Sydney (33ºS 59', 151ºE 14'). Replicate devices (*n* = 10) were wedged in crevices and between boulders in barren-grounds (Figure 1b). Approximately 50 devices (containing up to 200 abalone) can be wedged in the habitat by one diver in a one-hour dive. Devices were left for two weeks during which time an intense storm hit Sydney and devices were subject to heavy seas (up to 9 m swell, winds to 30 km). After two weeks, it was observed that devices had been affected by these conditions, but were not dislodged from their positions; all devices remained wedged in the habitat.

For the device to offer a suitable habitat for 'acclimatisation' it must have no adverse effects on abalone and must allow them access to their natural habitat. To assess the response of abalone to the device, we compared survival of abalone seeded into: (i) 'open' devices (the real device) and (ii) 'closed' devices with flywire wrapped around the PVC tube inside the cage. In the latter devices, water-flow was not restricted, but abalone were prevented from leaving the device by the fine-mesh flywire. It was predicted that if the device had no adverse effects on the survival of juveniles, then survival would be similar in open and closed devices. In the same experiment, we also assessed whether *H. rubra* can move from the open devices by recording their distribution in and outside of the devices over five days. Abalone shells were tagged with permanent markers of different colours, so the treatment from which they came (open vs closed) could be determined once outside of the tube. Replicates of each treatment (*n = 5)* were interspersed among two large aquaria. *H. rubra* of the same stock were left untouched in adjacent aquaria under the same environmental conditions which served as a control: 100% of these abalone were alive after five days. These data are summarised in Table 1. After five days, only three abalone had died from the total seeded in all tubes; one from open devices and two from closed devices. This suggests that the devices have no short-term adverse affect on the survival of *H. rubra*, even when they are trapped inside. Most (91%) of the abalone moved out of the open devices within 24 hours. After five days a few abalone were found inside open devices which were not previously recorded inside devices (Table 1), suggesting that *H. rubra* may move out and in of open devices over short periods of time and therefore would not suffer from confinement in the devices.

These results suggest that the device meets the criteria for a successful seeding substrata, so in the following field experiments, *H. rubra* were seeded directly into these devices and kept in flowing seawater at the NSW Fisheries Aquaculture at Port Stephens. Flywire was wrapped between the tube and the cage, to maintain required densities inside each tube until seeding. To seed abalone, devices were taken as described to natural habitats in catch bags using SCUBA. Once underwater, the flywire was removed and the tube placed back inside the cage, which was folded closed. The devices were then firmly wedged in between boulders in appropriate habitat.

Study site

Sites were chosen around Fingal Head and Fingal Island (from 32ºS 45', 152ºE 10' to 32ºS 44', 152ºE 11'; Figure 2). A few adult abalone were observed in some of these sites, but they were not counted, nor their identity distinguished as *H. rubra* or *H. coccoradiata*. Sites were selected based on the type of habitat that was present and the physical conditions. More sheltered sites were

preferential because conditions would be more favourable for repeated sampling. Sites were selected because of the presence of boulder habitat, barren of macro-algae and where rocks where covered with encrusting coralline algae; the major diet of juvenile abalone (Shepherd and Cannon 1998, Shepherd and Turner 1985). Adjacent habitat consisted of forests of macroalgae: predominantly *Ecklonia radiata*, *Phyllospora comosa* and *Sargassum* spp. with understorey of articulated red algae. Although not quantified, known predators of juvenile abalone were observed including brittle stars, sea stars, crabs, lobsters, octopus, wrasse and morwongs. Sites were chosen to be from 5 – 10 m in depth, because shallower depths have been shown to reduce survival of abalone seed (McShane and Naylor 1995) and *H. rubra* are not frequently found at deeper depths in NSW (McShane 1999).

General sampling and experimental methods

A major aim of this project was to assess whether seeding blacklip abalone at small densities could result in adequate survival. As a result, all of the hypotheses tested in the following experiments involve seeding *H. rubra* at densities of approximately 100 – 120/m^2, depending on supply from the aquaculture. Experimental plots were to be an area of 1 m^2 around the device(s). They were permanently marked using fluorescent orange barrier-mesh made from heavy duty plastic. Sections of mesh were wrapped around boulders and secured with cable ties. Juvenile abalone have been reported to move no further than about 2 m in the first couple of months (Shepherd *et al.* 2000), so plots were placed > 4 m apart to ensure independence from each other. An area of 2 m^2 was sampled including the central 1 m^2.

Sampling was done using timed searches (McShane 1995b, Shepherd and Partington 1995) which has been shown to provide precise estimates of abundance (McShane 1994). The diver would locate the centre of a plot and swim radially outwards to cover the total area (2 × 2 m) searching for 5 minutes in cracks, crevices and carefully under boulders for the hatchery-reared abalone (noticeable turquoise shells). Previous experiments sampling in similar habitats for seeded abalone showed no increase in number found after 5 minutes of searching 2 m^2 (unpublished data). More destructive sampling or suction sampling (e.g., Heasman *et al.* 2004) could not be done because we wanted to assess survival through time in replicate plots. Resources were insufficient to do more replication which would allow independent, and destructive sampling through time of replicate plots.

Effect of spatial and temporal arrangement of seed

To investigate whether survival differed depending on how abalone were seeded in space and time, we seeded the same density of *H. rubra* (120 m^{-2}) in four different configurations: (i) 'clustered': all abalone in one tube, (ii) 'clustered devices': abalone divided among 6 devices (20 abalone/device) with the devices themselves clustered into two groups of three, (iii) 'dispersed': abalone divided among 6 devices with the devices dispersed in space, and (iv) 'temporally dispersed': abalone divided among 6 devices with the devices deployed through time and in space (Figure 3). Devices were seeded with *H. rubra* at appropriate densities (100 or 20 per device) in aquaculture. Four replicates of each plot were interspersed in barren boulder-grounds at each of two sites at Port Stephens (Figure 2; S1, S2). After initial sampling (to 2 weeks), it was evident that the amount of effort required to deploy abalone in the 'temporally dispersed' configuration was not matched by any increased benefit in survival relative to the other treatments. This configuration was therefore not deployed at Site 2, and the number of replicates for the other treatments could be increased to *n* = 5 at Site 2. After 1 month, no abalone were found inside devices so these were removed from plots to prevent litter.

The experiment was set up in Austral winter 2004 and sampled at weekly, fortnightly, monthly and two-monthly intervals (allowing for swell and conditions) up to 6 months. So that we could analyse for differences in survival among treatments over time, we set up two extra sets of replicates for the

clustered and dispersed arrangements. One set was sampled at 24 hours, the other at 48 hours and these could then be independently compared to the set of plots remaining at 8 days. We also measured the length of seeded abalone after 1 month using a metal compass to assess their growth.

Use of sea urchins to promote survival of abalone

To assess whether adult urchins, *Centrostephanus rodgersii*, could act as a 'natural' seeding device, we seeded a small density (120 m^{-2}) of *H. rubra* 'under' or 'near' adult urchins in two spatial configurations within 1 m^2 plots: (i) 'clustered': 60 abalone put under and near each of 2 urchins, (ii) 'dispersed': 20 abalone under/near each of 6 urchins. For this experiment, abalone were seeded onto flywire pouches at the appropriate densities and stored in flowing seawater at the NSW Fisheries Aquaculture, Pt Stephens. To seed abalone under urchins, urchins were lifted off the substratum with a hook and the abalone emptied from the pouch into the underlying crevice. The urchin was then held back in place until it re-attached to the substratum. To seed abalone near urchins, abalone were emptied from the pouch into crevices or cracks within the radius of the urchin's spines.

This experiment was set up during Austral winter 2004 at two sites of barren boulder-grounds at Port Stephens (Figure 2; S3, S4). Plots were chosen with a density of *C. rodgersii* of at least 6 m^{-2}. Initially, eight replicates were set up, but more than one replicate was lost from a few treatments in heavy swell, so the number of replicates for each treatment was reduced from 8 to 6. To balance the design, replicates from treatments with more than 6 replicates were randomly removed from the analyses. Plots were interspersed at each site with a distance of 2 m between each plot and were marked using plastic barrier-mesh. Plots were sampled after two weeks, monthly and then at two-monthly intervals up to 6 months. At 6 months, no abalone were found in any plots so results are presented for up to 4 months. Sampling was again done using 5 minute time searches in a total area of 2 m^2 for each plot. Crevices, cracks and the undersides of boulders were searched where possible and a minimum of six urchins were randomly overturned to search the underlying area. It was noted whether *H. rubra* were found associated with *C. rodgersii*.

Survival of abalone fed artificial vs natural diet

To test the model that survival of *H. rubra* is different depending on whether they are fed on natural diet (reddish shell) or artificial diet (bright turquoise shell) we seeded artificially and naturally fed abalone to natural boulder-grounds and recorded their survival through time. Natural diet consisted of a mixture of algae and results in the natural brick-red shell of *H. rubra*, in contrast to the turquoise shell which results from the usual artificial diet. Any differences in measured survival of these two types could be due to variation in recognition by divers, so before seeding, we assessed whether turquoise shells were more easily recorded *in situ* than were red shells. We used empty shells weighted with small lead sinkers glued to the underside with epoxy. Natural, small red shells were unavailable, so we painted turquoise shells with terracotta coloured paint watered down to create a more realistic mottled effect and allow the shell ridges to appear (Figure 4). For convenience we did the experiment at Bear Island, Sydney (33ºS 59', 151ºE13'). A known number (6 – 10) of abalone were hidden in crevices and between boulders in rocky-reef with kelp (*Ecklonia radiata*) and without kelp (*n* = 5 for each colour of shell). Another set of divers then searched these areas for 5 minutes without prior knowledge of the number hidden. Different amounts of abalone were seeded into each plot to avoid divers learning the amount hidden in plots. No significant interaction was detected between the percent of turquoise vs red shells found in kelp vs boulder habitat (ANOVA: $F_{1, 16} = 0.06$, $P > 0.05$) suggesting that the ability of divers to detect shells of different colour did not vary (ANOVA: $F_{1, 16} = 0.81$, $P > 0.05$) and is not different between kelp and boulder habitats (ANOVA: $F_{1, 16} = 2.23$, $P > 0.05$).

Our original hypotheses predicted differential survival between *H. rubra* fed on different diets in habitats with macroalgae vs barren habitats, because of variation in levels of camouflage.

Unfortunately, lack of animals prevented this experiment from being done in more than one habitat and at more than one site. Nevertheless, *H. rubra* were re-introduced at a density of 100 m^{-2} in barren boulder fields at one site at Port Stephens (Figure 2; S5). Fifty abalone were seeded per device and devices were deployed in a slightly modified 'clustered' arrangement based on results from the first experiment; two devices were deployed in each plot (*n* = 5 for each shell colour). Plots were again interspersed in the habitat, marked and separated from one another as described for the other experiments. Sampling was done initially after two weeks and at 5 months because results from the previous experiments showed no differences in patterns of survival with more frequent sampling. Sampling methods were the same as described for the other experiments and all divers were made familiar with both shell colours before sampling.

Analysis of data

The number of *H. rubra* found in each experiment was compared among treatments for each time-interval using analysis of variance (ANOVA). Problems with assuming that recovery is survival are discussed. To test for differences among survival of *H. rubra* seeded in different arrangements, one-way ANOVAs were done with 'arrangement' as a fixed factor with 4 levels: clustered vs clustered devices vs dispersed vs temporally dispersed (*n* = 4) at Site 1 and clustered vs dispersed vs temporally dispersed (*n* = 5) at Site 2. Where extra replicates for dispersed and clustered arrangements were independently sampled for three time periods (Site 1), a two-way ANOVA was done with the three levels of 'time' (1, 2 and 8 days) (random factor) nested within two levels of the 'arrangement' (clustered vs dispersed) (fixed factor) (*n* = 4).

To test for differences in survival of *H. rubra* seeded near and under urchins, and whether survival differed according to spatial arrangement, two-way ANOVAs were done in which 'arrangement' (clustered vs dispersed) was treated as a fixed factor orthogonal to 'proximity' to urchins (near vs under; a fixed factor; *n* = 6). To test for differences in survival of *H. rubra* fed on different diets, one-way ANOVA was done in which 'diet' was treated as a fixed factor (*n* = 5).

Each analysis was done separately for each time at each site because replicates within sites were not independent among sampling intervals, and sampling intervals were slightly different among sites within each experiment.

4.2.1.4.   Results

Within days, the total recovery among treatments within any experiment ranged from 7.6% to 30.6% of the number seeded. After two weeks, this figure dropped to 0.6% – 5.5%. After 1 – 2 months, total apparent survival was again reduced, but became consistent during this time at between 0.6% – 1.8%. After 6 months, apparent survival was reduced again to between 0% – 0.6%. Mortality as estimated by the number of empty shells in the sampled area, was never greater than 0.4%, at any site, at any time, for any experiment.

Effect of spatial and temporal arrangement of seed

The raw data (Figure 5) show that the percentage apparent survival of *H. rubra* was greater when seeded in clustered than any other arrays within the first week or so at each site. Indeed, two-way ANOVA detected a significant difference in the number of abalone that apparently survived between clustered and dispersed arrangements regardless of time since seeding (1, 2, 8 days) at Site 1 (ANOVA: $F_{1,4}$ = 12.60, $P < 0.05$). SNK tests showed that a greater number of abalone apparently survived when seeded in clustered rather than dispersed arrays. Apparent survival of abalone was also significantly greater in clustered than in dispersed arrays after 2 days at Site 2 (ANOVA: $F_{1,8}$ = 5.72, $P < 0.05$). After 8 days, the least apparent survival was recorded for plots in which devices had been clustered at Site 1 and the least survival was found where devices were dispersed at Site 2

(Figure 5). No significant differences were detected between seeding arrangements from 8 days onward (Table 2).

There seem to be substantial differences in the number of abalone found between the two sites (Figure 5). After 2 days, total apparent survival across all treatments was up to 24.9% at Site 1, whilst only 7.6% were found at Site 2. Total apparent survival at each site was reduced after 1 month and became consistently similar between the 2 sites (1% at Site 1 and 0.7% at Site 2). Total apparent survival remained between 0.4% – 0.8% over a total of 6 months (Figure 5). The lowest recorded recovery was at Site 2 after 5 months, where only 1 individual was found. These values correspond to 7 – 20 individuals found from the 1920 seeded at Site 1, and 1 – 13 individuals found from the 1200 seeded at Site 2, from 1 – 6 months. At 6 months, the mean recovery was 1 individual per m^2 for both sites.

Mortality was not substantially different among treatments, nor was overall mortality different among intervals of sampling. There was a greater total mortality over the course of the experiment at Site 2 (0.7%) than at Site 1 (0.3%), which may correspond with smaller numbers recovered in total at Site 2.

The mean shell-length of *H. rubra* of all individuals found across all treatments at Site 1, increased from 11.8 (+S.E. 0.3) to 13.4 mm (+S.E. 1.0) after 2 months and to 19.8 mm (+S.E. 1.6) after 6 months (Figure 6). Large variation in the number of individuals found between treatments prevented reliable comparison of growth among treatments. For example, the greater shell-length in clustered treatments (Figure 6) does not necessarily imply that all abalone from this treatment grow more than the others, but that more individuals were found and could be measured from this treatment. There was, nevertheless, an average increase in shell length by 7.8 mm of seeded *H. rubra* recorded in this experiment after 6 months.

Use of urchins to promote survival of abalone

No interaction was detected between the proximity to urchins at which abalone were seeded and their seeding arrangement, at either site at any time (Table 3). Data were therefore pooled to display apparent percentage survival under vs near urchins, regardless of configuration (Figure 7; *n* = 12). For most time intervals, these data show greater apparent percentage survival near rather than under urchins at Site 1, but the reverse pattern at Site 2. No significant differences in the apparent survival of *H. rubra* between seeding under vs near *C. rodgersii* were detected using two-way ANOVA at any time intervals at Site 1 or Site 2 (Table 3). The exception was one case at Site 2 after 12 days, where a greater percentage of *H. rubra* were found where they had been seeded under urchins compared to near urchins (ANOVA: $F_{1,20} = 5.23$, < 0.05, SNK tests; Table 3). After a few days, the apparent mean survival where abalone had been seeded under urchins ranged from 10.8% at Site 2 to 11.3% at Site 1. Apparent survival where abalone had been seeded near urchins was 11.74% at Site 2 to 15.6% at Site 1. Large differences in survival between treatments disappeared at 1 month.

Data were also pooled to display apparent percentage survival in clustered vs dispersed arrangements seeded in associated with urchins, regardless of proximity (Figure 8). These data suggest that after a few days, there was slightly greater apparent survival when abalone were seeded in clustered (mean survival 16.1%) rather than dispersed arrays (mean survival 10.7%) at Site 1, but this pattern was opposite at Site 2 (at least for the first two intervals) (Figure 8). Mean apparent percentage survival in dispersed arrays was 12.2% vs 10.7% in clustered arrays at Site 2. These differences among treatments seemed to disappear after 1 month at both sites and mean apparent survival for any treatment across sites ranged from 0.3%-2.5%. Furthermore, no significant differences in the apparent survival of *H. rubra* between clustered and dispersed arrays were detected using two-way ANOVA at any time intervals at either site (Table 3).

Whilst patterns between proximity to urchins and the seeding arrangement in association with urchins (as described above) were not consistent between sites, the total apparent percentage survival across all treatments was not substantially different between sites (Figure 7, 8). As is the case for the first experiment, a relatively large number of abalone were recovered within days: 11.3%-13.4% of the total seeded regardless of treatment, but this value dropped to between 4.1% and 5.5% after 1 week. Differences between apparent survival of *H. rubra* in response to proximity to urchins and spatial arrangement disappear with a longer time since seeding. The total apparent survival at 1 month across all treatments was 1.8% at Site 1 and 0.6% at Site 2, corresponding to 51 and 16 individuals found respectively of the 2880 planted at each site. Total apparent survival across all treatments up to 2 months was 0.4 % at Site 1 and 0.6% at Site 2. A mean density of $1/m^2$ after 6 months was recorded for any seeding arrangement in association with urchins. After 5 months, Site 1 was sampled with only 1 abalone recovered and after 8 months Site 2 was sampled with no abalone found. Never more than 3 abalone shells were found at any time of sampling across all treatments and the total mortality based on recovered shells was 0% at Site 1 and 0.2% at Site 2.

To assess whether abalone remained in association with urchins after seeding, we recorded whether abalone were found under an urchin or in surrounding spaces between boulders (Figure 9). There seemed to be a greater proportion of abalone found still associated with *C. rodgersii* when they were seeded near rather than under urchins at Site 1. ANOVA only detected a significant difference in the proportion of abalone still associated with urchins after 1 day ($F_{1, 23} = 4.85$, $P < 0.05$), but not at 7 days ($F_{1, 23} = 0.28$, $P > 0.05$). This pattern was not, however, spatially general: a greater proportion of *H. rubra* were found still associated with urchins when seeded under vs near at Site 2 (Figure 9). Again, ANOVA only detected a significant difference at one time period, 12 days ($F_{1, 23} = 7.66$, $P < 0.05$) at Site 2. Nevertheless, a greater percentage were found not associated with *C. rodgersii*, but these were, in all cases, found in cracks and crevices in the surrounding 2 m^2.

Apparent Survival of abalone fed on artificial vs natural diet

After 18 days, significantly more abalone were found which had been fed on artificial diet than of those fed on natural diet (Figure 10; ANOVA: $F1, 9 = 9.34$, $P < 0.05$). We recovered no empty shells from this experiment. After 119 days, only 1 abalone was found and no differences were evident between the two diets at this time. The experiment was terminated because data were restricted to one site only and, despite results from our pilot study, there seems to be confounding between the number recovered and the ease at which shells of different colour can be seen *in situ*.

4.2.1.5.   Discussion

Apparent survival of juvenile *H. rubra* was variable among sites and survival after 6 months was small, but approximately matched the natural density ($1/m^2$) previously observed for wild *H. rubra* of this size and age (SL 15 – 35 mm and 1 year old) . Our experiments show that variation in the spatial arrangement of blacklip abalone and their proximity to *C. rodgersii* is of little consequence to their apparent survival. Differences among treatments in each of our experiments, observed in the first few weeks, were spatially variable and did not persist through time. Schiel (1993) also reported substantial spatial variation in the survival of *Haliotis iris* in New Zealand. Generally, the differences in our study disappeared after 1 month and total survival was between 0.6% – 1.8%. Total survival became relatively constant at each time of sampling after 1 month up to 6 months, at 0.05% – 0.6%. A main feature of the current study was that attempts to re-seed small juveniles of *H. rubra* in small numbers can result in survival to match natural adult populations observed in NSW. Therefore, large numbers of juveniles probably do not need to be seeded to increase the rate of recovery of depleted populations.

Apparent survival was not increased by dispersing abalone in space or through time. Whilst larval mortality and growth can be strongly density-dependant (McShane 1991), the current study adds to

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recent works in southern Australia and New Zealand, that show mortality of juveniles may not be density-dependant when the number seeded is below $100/m^2$ (Shepherd *et al.* 2000, the current study). Seeding and diver activity may attract opportunistic predators and scavengers (Tegner and Butler 1989), but in the current study, dispersing seed, either though space or time (or both) did not substantially reduce mortality. When we could detect differences in survival among seeding arrangements, survival was often greatest where abalone had been clustered in one device rather than any dispersed method. Schiel (1993) has shown that substantial mortality can occur when abalone are clustered into tubes, but up to 500 abalone can be safely transported and, as evident from the current study, successfully seeded into natural habitat. Seeding *H. rubra* in numbers which reflect those that occur naturally may overcome problems associated with density-dependant mortality. This constituted an alternative, complimentary experimental strategy in which small (mean SL ~9 mm hatchery produced juvenile abalone were seeded at such sparse densities ($8/m^2$) over large areas ($1000 m^2$) of juvenile habitat (See Section 4.2.2 for summary).

Mortality is often greatest soon after seeding, often because abalone are vulnerable to predation when acclimatising and seeking appropriate microhabitat (Tegner and Butler 1985). In the current study, abalone were found living inside seeding devices up to 18 days after seeding, suggesting that the device successfully sheltered *H. rubra* from predators whilst they acclimatised to their surroundings. Few successful predation attempts were observed when abalone was seeded inside the devices and with urchins, compared to pilot studies in which *H. rubra* were hand planted in crevices and were quickly attacked. In addition, we recovered very few empty shells in the first few sampling dates, nor at any time thereafter. Most abalone moved from the seeding device into natural microhabitat within a few weeks (Figure 11a), a similar result to other studies which have used artificial structures (Davis 1995) or seeding modules (McCormick *et al.* 1994). Schiel and Weldon (1987) gave evidence that abalone were successfully acclimatised after a period of several days and exhibited behaviour similar to that of their wild counterparts. Often seeding substrata are used purely as a means of reducing stress from handling and modules which lack predator protection often infer no advantage (Shepherd *et al.* 2000). Our results suggests that handling stress is reduced by using devices, they act as a good stepping-stone habitat, and we stress that mortality may be further reduced if the seeding strategy incorporate some protection from predators (e.g., devices or the use of urchins).

Our results suggest that seeding *H. rubra* by hand under urchins may be a successful strategy. Despite reports of sluggish behaviour of hatchery-reared juveniles from laboratory studies, Tegner and Butler (1989) observed that abalone would rapidly turn over, attach and move into crevices after planting. We similarly observed that when *H. rubra* were seeded within the radius of the spines of an urchin, they would rapidly right themselves and move further under the urchin, or into the crevice below (Figure 11b). There was no advantage to seeding under urchins rather than planting seed within the radius of their spines, so we suggest the latter as a potential method because it was much quicker and at least 50 urchins could be 'seeded' by one diver in one dive. Little is known about the potential interactions between other gastropods sheltering under urchins (e.g., Day and Branch 2002, Chapman and Underwood 2003) and the seeded abalone, and whether these are likely to negatively or positively affect abalone. We observed small proportions of abalone still in association with urchins up to 4 months (Figure 10b), but the majority of individuals we recovered were hiding in crevices and between boulders without urchins. It is known that adult abalone and *C. rodgersii* are negatively correlated in NSW (Andrew and Underwood 1992), so further work needs to be done to assess whether juvenile *H. rubra* seeded under urchins survive in the long-term to form part of the adult population.

Given the lack of research about the ecological interactions of the two species for both juvenile and adult abalone, at this point, we advocate the use of an artificial seeding module. Despite being more time-consuming to construct, the module infers several other advantages over using urchins. First, urchins can move up to 2 m within 24 hours (Bernat 2005), yet a stationary device when placed

suitably allows juveniles plenty of time to seek their own microhabitat within the reef after acclimatisation (our devices were recorded *in situ* up to 4 months). Second, because abalone are planted already in the tube, any differences in behaviour from handling stress (e.g., Schiel and Weldon 1987, Tegner and Butler 1989) can be overcome whilst still being protected inside the tube. Third, the devices are more easily transportable than the mesh pouches used when seeding with urchins, and can be more easily kept cool and moist to reduce stress from transport without danger of crushing the abalone. Finally, the devices can be placed in a number of habitats (e.g., kelp forests, barrens, fringe habitat) and do not rely on the persistence, abundance, or distribution of another organism. Preliminary data suggest survival of juvenile seed may be slightly greater when seeded in the same habitat in devices than under urchins (Goodsell *et al.* in prep.).

Predation is thought to be a substantial cause of early mortality of seed (Tegner and Butler 1985, Shepherd *et al.* 2000). Mortality from predation can often be identified by distinct markings on the shells (Hines and Pearse 1982, Tegner and Butler 1985) and recovery of these shells can provide an estimate of mortality. In the current study, however, empty shells accounted for less than 0.7 % of the amount seeded in any experiment at any time, suggesting predation is unlikely to be a major source of mortality. We do acknowledge that predation attempts on small shells often fragment them beyond recognition (Schiel and Weldon 1987), so our estimate of mortality from predation is likely to be an underestimate. The intact shells we found more probably represent death from planting or other causes and it is promising that the figure is negligible. Furthermore, the number of dead shells found soon after planting was not substantially greater than that found afterwards, suggesting low levels of mortality associated with planting. Schiel (1993) argued that predation risk to abalone was not as dramatic in New Zealand as was the case in California (e.g., Tegner and Butler 1989), which may also be true for Australia. Regardless, it is likely that predation was minimal because adequate protection from predators was given either in devices or with urchins.

Schiel (1993) suggested habitat destruction as a major cause of mortality in *Haliotis iris* after seeding in New Zealand. Shifting of nearby sand and burial of juvenile habitat was an identifiable source of mortality in his study. At a few of our sites, we also observed burial of some plots by sand in which we consistently did not find abalone. Unfortunately, the nature of the reef at Port Stephens consists of rocky reef stopped short about 5 – 7 m offshore by sand, so burial could not have been prevented. Nonetheless, we also found seeded juveniles under boulders in partially sandy substrata, so although burial may be a cause of mortality it is unlikely to be a major factor in the current study. Similarly, Tegner and Butler (1989) reported that *Haliotis rufescens* were more abundant under rocks set in sand compare to rocks set on rock. Very shallow, wave exposed sites were shown to negatively affect the survival of *Haliotis midae* in South Africa (de Waal *et al.* 2003) and we similarly recovered less *H. rubra* from exposed vs more sheltered sites. Clearly, whilst generalisations can be made about the habitat requirements of abalone, different species of *Haliotis* can have different microhabitat requirements and further research would be well placed to understanding these for the different commercially harvested species of abalone in Australia, since little has been done to add to the work of Shepherd (1975) and Shepherd and Turner (1985).

The quality of habitat (e.g., food availability) is also cited as a factor limiting the survival of abalone seeded into natural habitat (Tegner and Butler 1989). All *H. rubra* that were recovered were observed feeding with fully spread mantles and we recorded a mean increase in shell-length from some recaptures of 7.8 mm in 6 months. Such growth is well within the range of growth reported for wild *H. rubra* and another southern Australian species *H. laevigata* (Shepherd and Hearn 1983, Shepherd 1988). These results suggest that the survival of *H. rubra* seed was not limited by poor habitat quality and food availability. We recommend that cobble and boulder-grounds covered with encrusting coralline algae, adjacent to kelp beds are suitable for seeding small numbers of juvenile abalone. The scope of this study did not permit assessment of survival across more than one type of habitat. Studies such as Schiel (1993), where much care was taken to

select good quality, habitat are a good example of what needs to be considered in future experiments to assess the best habitat for seeding *H. rubra*.

It is likely that our small estimates of survival more accurately reflect our inability to find abalone. The cryptic behaviour of juvenile abalone makes it very difficult to accurately estimate their abundance and recovery may be a poor indicator of survival (Rogers-Bennett and Pearse 1998). Furthermore, abalone are often found in clumped distribution (McShane 1995b), which makes estimates of their abundance based on random sampling difficult. In the current study, abalone were often found in clumps under a boulder or urchin, a similar result to that shown by Ebert and Ebert (1988). The complexity of the habitat in which abalone are desirably seeded can cause variation in the recognition of abalone among divers and unreliable estimates of survival (McShane 1998). In natural surveys, often the smaller (< 20 mm) individuals are under-estimated (McShane and Smith 1991) which further complicates making accurate estimates of the survival of small seeded juveniles. Unaccounted-for abalone plague reseeding attempts all over the world (reviewed in Tegner and Butler 1989, McCormick *et al.* 1994) and it is a common hindrance to analysing the success of reseeding programs and experiments. That we recovered greater amounts of abalone within days to weeks of seeding, which then declined to a more constant level after 1 month, suggests that whilst abalone were still seeking shelter sites, shortly after seeding (Schiel and Weldon 1987), they were easier to sample. This is further supported by constant but smaller recovery of abalone from 1 – 6 months, when abalone were likely to be well hidden in crevices and cracks between boulders (Shepherd and Turner 1985).

Extensive analysis of sampling methods has been done by McShane (1994, 1995b, 1998) and Shepherd (1985), but there still exists much debate about the most accurate and precise method. Here, we followed their recommendations for precision and efficiency using timed searches, but it may be that monitoring has to cpmtomie for long enough to sample the seed once they become emergent (> 2 years; Shepherd 1975, Shepherd and Turner 1985). For example, Schiel (1993) found greater numbers of abalone at 2 yrs than 1 yr and concluded that measures of survival are inaccurate when abalone are small and cryptic, but recovery rates become better when sampled after the seed is older and become sedentary and emergent. It would seem prudent to develop seeding programs or further small-scale experimental work which has the scope to track abalone over a period of years and possibly use genetic evidence to find hatchery-reared qualities in captured stock (e.g., Gaffney *et al.* 1996).

Survival based on sampling of experimental plots and the immediate surrounding area does not account for potential larger-scale migration away from the study sites. This may be a likely cause of unaccounted-for seed. There was enough suitable habitat for *H. rubra* to move away from the sites in the current study, but there are a number of reports of planted abalone moving only small distances (2 – 3 m) within the time periods of the current study (Hines and Pearse 1982, Shepherd *et al.* 2000). We conclude movement out of study sites is an unlikely cause of mortality in this case.

As more research is done about the factors that affect survival of abalone seeded to natural habitats and transport and seeding techniques are refined, reseeding of hatchery reared abalone is cited as an increasingly economically favourable method to ensure natural stocks of abalone persist (EIS 2005, Schiel 1993). There is still much to be done and, despite world-wide attempts, Japan remains one of the few countries to report success consistently. For example, over thirty years ago, Bardach (1972) reported 10% survival of abalone seeded at a similar size to the current study after 2 – 3 years, with an annual catch of 100, 000 individuals. Comparing among reseeding strategies is difficult because of the range of techniques, numbers and species of abalone seeded, sampling techniques, lack of replication, different types of habitat used, and different temporal and spatial scales over which the programs are done. Results from California (Tegner and Butler 1989, Davis 1995), New Zealand (Schiel 1993) and Australia (Shepherd *et al.* 2000) report much lower survival than results from Japan. For example, Rogers-Bennett and Pearse (1998) found survival similar to

that of the current study (< 1%) after seeding 50, 000 abalone among six sites in California, but despite this low survival, one third of the sampled cohort of abalone in the study sites were seed.

Current reseeding in Japan is also successful, but nowadays they are able to grow seed to a larger size at less cost and can therefore plant larger individuals (> 30 mm), ensuring greater survival (Saito 1984). In Australia and New Zealand, it is only cost-effective to rear large numbers of abalone to a small size (the size used here) and it is hoped that seeding in large numbers will result in greater survival. Given the recent debate over density-dependant mortality of seed (Hilborn 1998) and predation (Tegner and Butler 1989, Shepherd and Clarkson 2001), it may be more beneficial to plant fewer numbers, as evident form the current study. Moreover, survival of seed is dramatically increased with the size of abalone (Inoue 1976, cited in Tegner and Butler 1989). Saito (1984) has recommended that seed be at least 20 – 30 mm before planting to ensure survival rates of > 30%. Pertinently, estimates of mortality of wild *H. rubra* show a marked decrease in annual mortality with increasing age in boulder habitat (Shepherd and Breen 1992). Obviously, there is a trade off between the cost of seed and the size at release, but in the current study we show that seeding a small number of abalone can result in enough survival (that we can measure) to match that of natural populations. Whilst button-size juveniles provide the most cost-effective seed size in Australia to date (Heasman *et al.* 2004, Schiel 1992), it may be feasible to rear abalone to a larger size, and seed fewer of them as a strategy for success.

Despite arguments that reseeding abalone is unviable (Tegner 2000, Hilborn 1998), recruitment of abalone may be increased by the presence of seeded abalone, hence eliminating the need for constant reseeding. We observed more recruits of wild *H. rubra* and *H. coccoradiata* in our study sites at the end of the experiment at a few sites, but cannot know if this was due to the presence of seeded abalone, or that this was during a period of recruitment. Davis (1995) found more wild abalone where he had seeded hatchery-reared abalone in artificial habitat than where artificial habitat existed without hatchery-reared abalone. This suggests that even if reseeding of hatchery-reared abalone does not substantially enhance depleted populations themselves, recruitment to natural populations from wild abalone may be cued by reseeding abalone. Prince *et al.* (1987) has shown that recruitment of *H. rubra* larvae is related to the density of adults, hence re-stocking to a level that we have shown may promote natural recruitment. Populations of abalone are patchily distributed (McShane 1995b, 1998) and, as suggested by the EIS for the NSW Abalone Fishery (2005), small-scale management of abalone sub-stocks may be more appropriate. Reseeding attempts done at similar scales to those done here, therefore have the potential to increase the rate of recovery of localised populations of blacklip abalone.

4.2.1.6.   *Conclusions*

Reseeding abalone may still be a viable option for enhancement of depleted abalone stocks in Australia. We matched natural densities by seeding small numbers of *H. rubra*: recovering a mean density of 1 individual m^{-2} at each site from a total of 1220 and 2880 seeded in devices and with urchins, respectively.

Seeding with numbers greater than this (with possibly smaller seed to match the cost) are unlikely to be any more successful (Heasman *et al.* 2004). There is much promise in these techniques in south eastern Australia (Shepherd *et al.* 2000), but much more research needs to be done to understand the factors that affect abalone and better ways to estimate survival and mortality and their causes before recommendations for commercial-scale seeding can be done.

Factors maintaining survival of juvenile abalone are not well understood (Shepherd and Breen 1992). Further directions for study include investigating the variation in the behaviour and abundance of predators, food availability and interactions among potential competitors. As an example, van der Meeren (2000) found that predators of hatchery-reared lobsters were more

abundant in winter and the type of substratum also affected predation. Moreover, variation in predation and predator abundance, changes in the typically dynamic rocky-reef habitat and the specific characteristics of each site, stresses associated with handling and conditions in the hatchery (e.g., diet), can all affect survival in different ways and they can interact. The magnitude of these effects and the relative importance of each is likely to vary, not only according to the specific characteristics of each site, but temporally as well. Similar small-scale experiments like the ones done here could be done in NSW to investigate the nature of predation and its variation to better plan a commercial-scale seeding effort.

The current study, through FRDC funding, has addressed a major objective of the EIS for the NSW Abalone Fishery (2005): the need for scientific evaluation of "small-scale experiments to further investigate the potential and effects of reseeding". We have identified that abalone can be successfully seeded in clusters and in association with urchins, but caution against interpretations that diet has an effect on survival because of the potential confounding aspect of diver-recognition of the different coloured shells. We have provided models about possible causes of small estimates of recovery evident in this and studies world-wide, and highlighted possible pathways for future research, including a rigorous examination of the potential ecological impacts of reseeding.

4.2.1.7.   Acknowledgements

4.2.1.8.   References

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Table 1.   Percentage survival and location (in or out of seeding device) of juvenile *H. rubra* seeded to 'open' or 'closed' devices in aquaria for 5 days. *n* = 5 devices per treatment seeded with an initial density of 15 abalone per device. Note that in some cases, abalone were not found in or out of the device (possibly escaped through the aquarium outflow), hence values do not total 100%.

| | 1 day | | | 2 days | | | 5 days | | |
| | Alive | | Dead | Alive | | Dead | Alive | | Dead |
	In	Out		In	Out		In	Out	
Open	0	91	0	0	73	0	1	97	1
Closed	97	1	1	99	1	0	99	1	1

Table 2.   Analysis of mean number of abalone in different seeding arrangements at separate intervals of time. Seeding arrangement was treated as a fixed factor with four levels at Site 1: clustered abalone vs clustered devices vs dispersed devices and abalone vs temporally dispersed devices and abalone (n = 4), and three levels at Site 2: clustered vs dispersed vs temporally dispersed (n = 5). No values were significant at P > 0.05, except * in which P = 0.0551.

Site 1	df	F	Site 2	df	F
8 days			**8 days**		
Arrangement	3	1.04	Arrangement	2	2.84
Residual	12		Residual	12	
18 days			**22 days**		
Arrangement	3	0.33	Arrangement	2	3.73*
Residual	12		Residual	12	
33 days			**42 days**		
Arrangement	3	0.04	Arrangement	2	0.10
Residual	12		Residual	12	
61 days			**63 days**		
Arrangement	3	1.14	Arrangement	2	0.50
Residual	12		Residual	12	
176 days			**161 days**		
Arrangement	3	1.17	Arrangement	2	0.40
Residual	12		Residual	12	

Table 3. Two-way ANOVA comparing the mean number of abalone recovered between dispersed and clustered arrangements seeded under and near urchins. Both seeding arrangement and proximity to urchins were treated as a fixed factors and orthogonal to each other ($n = 6$). No values were significant at $P > 0.05$, except * denoting $P < 0.05$. [†]denotes data were transformed to fourth root to homogenise variance (Cochran's C-test: $P > 0.05$).

Site 1				Site 2		
	df	F			df	F
1 day				**1 day**		
Arrangement	1	2.25		Arrangement	1	0.09
Proximity	1	3.57		Proximity	1	0.33
A × P	1	0.53		A × P	1	2.94
Residual	20			Residual	20	
7 days				**12 days**		
Arrangement	1	2.63		Arrangement	1	5.23*
Proximity	1	0.15		Proximity	1	0.80
A × P	1	0.02		A × P	1	0.00
Residual	20			Residual	20	
21 days				**35 days**		
Arrangement	1	0.82		Arrangement	1	1.10
Proximity	1	0.96		Proximity	1	0.07
A × P	1	3.48		A × P	1	1.72
Residual	20			Residual	20	
41 days[†]				**114 days**		
Arrangement	1	0.01		Arrangement	1	1.10
Proximity	1	0.53		Proximity	1	0.07
A × P	1	1.11		A × P	1	1.72
Residual	20			Residual	20	
62 days[†]						
Arrangement	1	0.04				
Proximity	1	1.00				
A × P	1	0.04				
Residual	20					
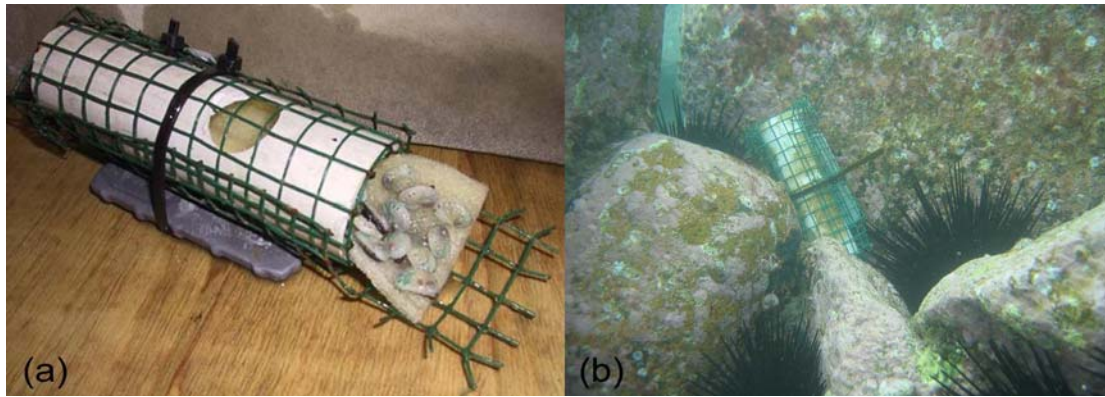
Figure 1.   (a) The device used to seed juvenile abalone to natural habitat. Note the large access holes and cage flap as detailed in the text. Abalone in this example were seeded onto sections of moist foam which were then slipped inside the device. (b) The device in situ.
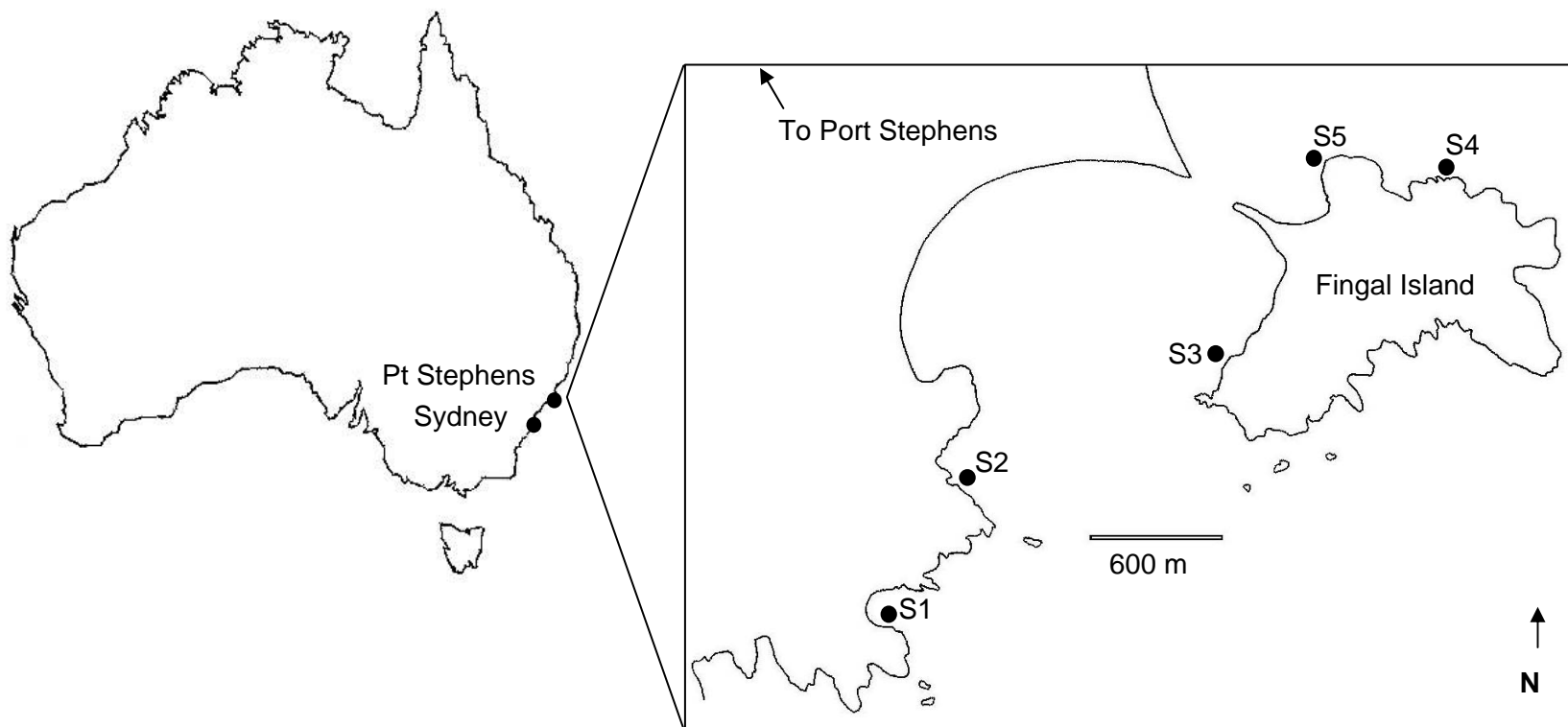
Figure 2.   Study sites (S1 to S5) for the three different experiments at Port Stephens, NSW.
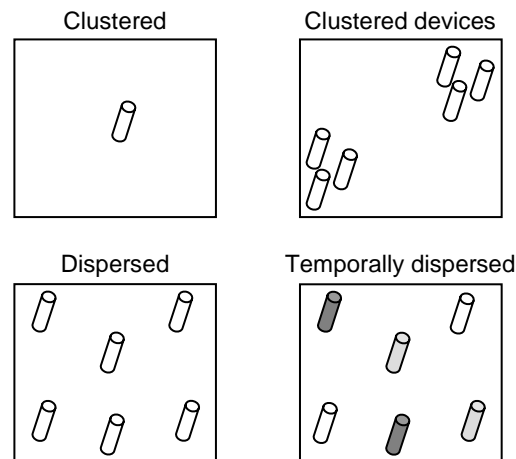
Figure 3.   Spatial and temporal arrangements of juvenile blacklip abalone and seeding devices. Each plot was 1m2. Lightly shaded tubes in the temporally dispersed arrangement were deployed after 1 day, the darker shaded devices were deployed after 2 days. Abalone were seeded at a density of 120 m^{-2} so that 120 abalone were seeded in one device in the clustered treatment, whilst 20 abalone were seeded in each of 6 devices in the other arrangements.

Figure 4.   Turquoise colour of *H. rubra* shell when fed an artificial diet (left), fed a natural diet (middle; the abalone was only fed a natural diet for the first stages of its life, so the natural red colour is only at the spire and the top part of the shell), and the shell-colour used as a proxy for natural diet in preliminary experiments (right).
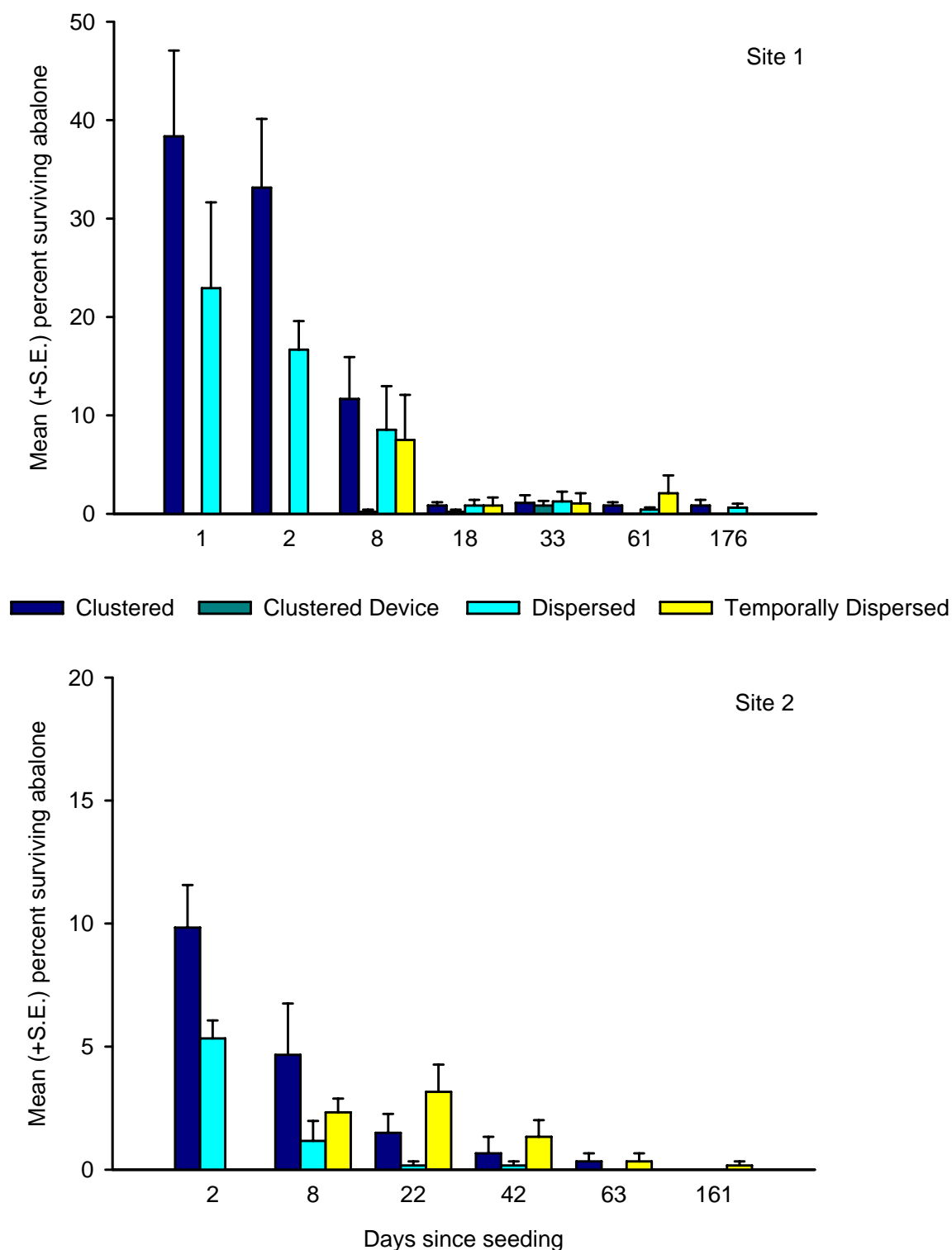
Figure 5. Mean (+S.E; $n = 4$ at Site 1; $n = 5$ at Site 2) percentage of *H. rubra* found in devices and surrounding 2 m^2 when seeded in clustered, clustered devices, dispersed or temporally dispersed arrangements at two sites at Port Stephens. Clustered devices were not deployed at Site 2 and temporally dispersed devices could not be sampled on days 1 and 2 at either site. Note the difference in scale on the y axis between Site 1 and 2.
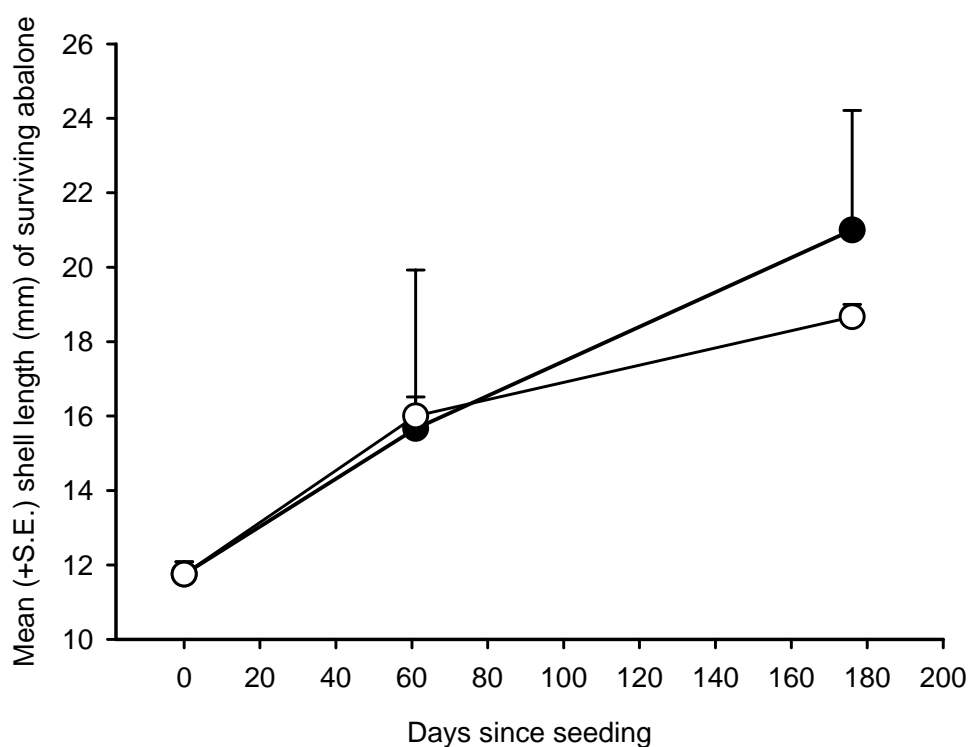
Figure 6. Mean (+S.E.) shell-length (mm) of abalone seeded in devices in clustered (filled circles) and dispersed (open circles) arrays. Length measured after 61 (clustered *n* = 3; dispersed *n* = 2) and 116 days (*n* = 3 for both arrays). Values for time zero are based on a sample from the initial stock (*n* = 5).
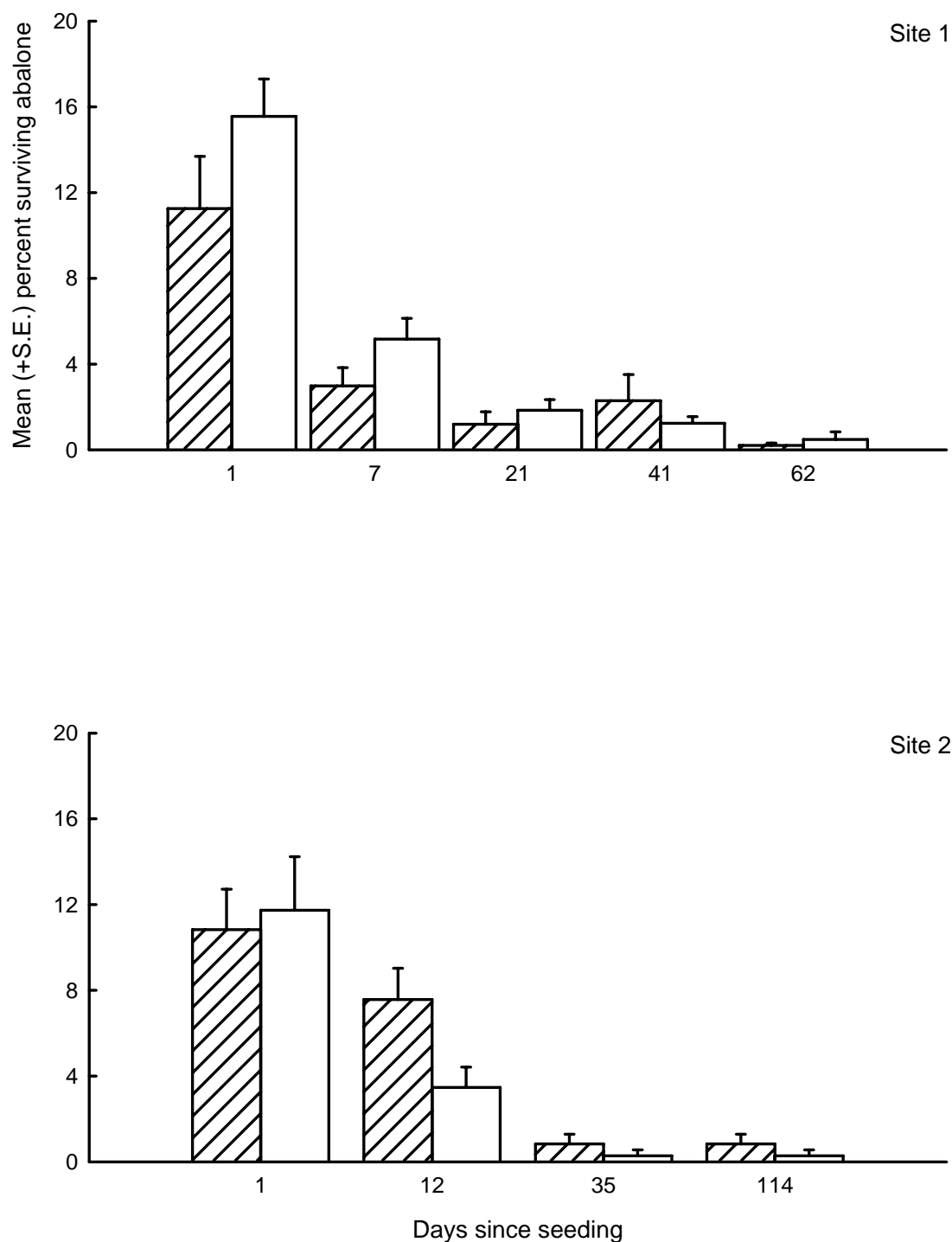
Figure 7. Mean (+S.E.; $n = 12$) percentage of *H. rubra* found when seeded under (striped bars) and near (open bars) *C. rodgersii*, regardless of the spatial configuration at which they were seeded, at two sites at Port Stephens.
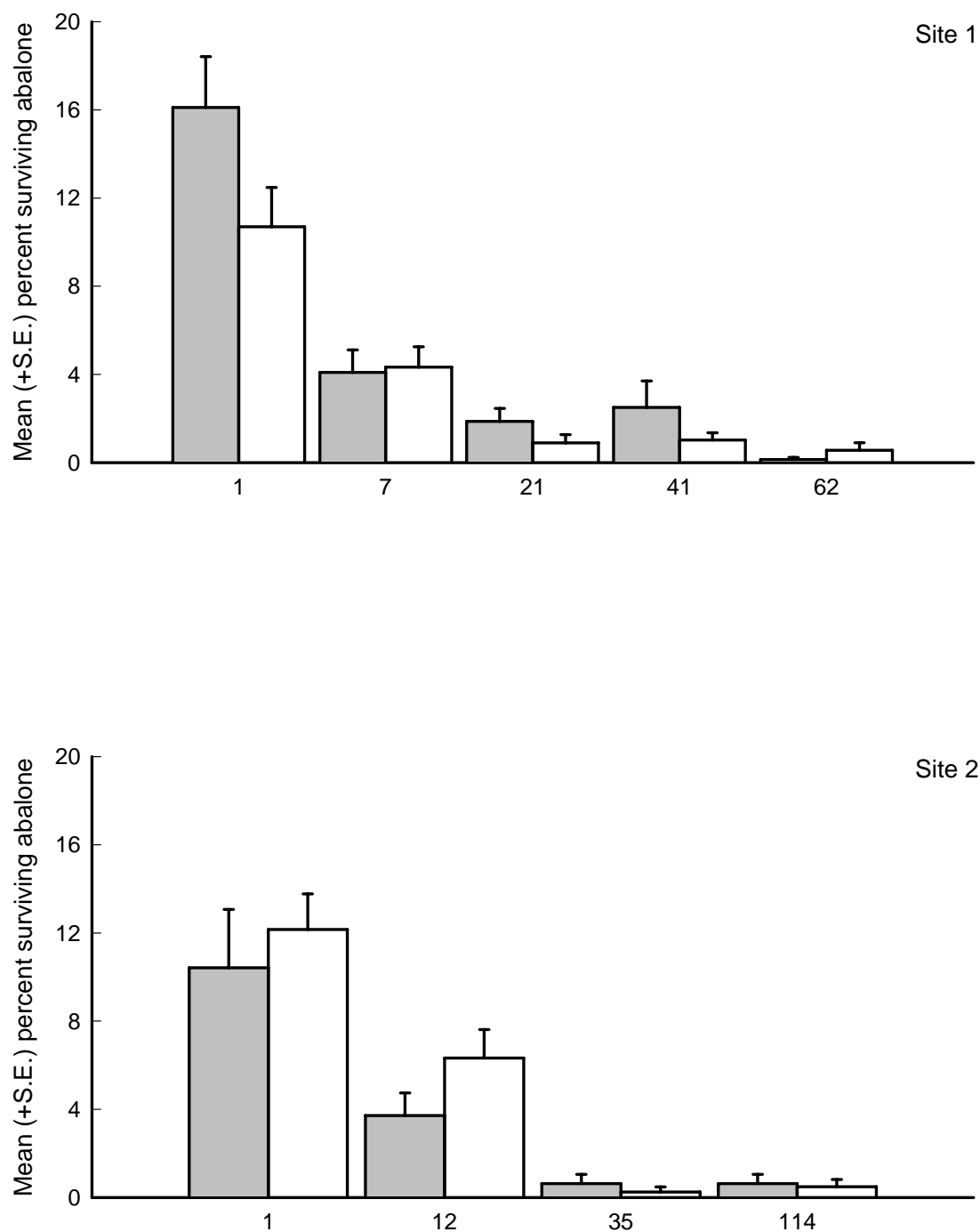
Figure 8.   Mean (+S.E.; *n* = 12) percentage of *H. rubra* found over time when seeded in association with *C. rodgersii* in dispersed (shaded bars) or clustered (open bars) arrangements, at each of two sites at Port Stephens.
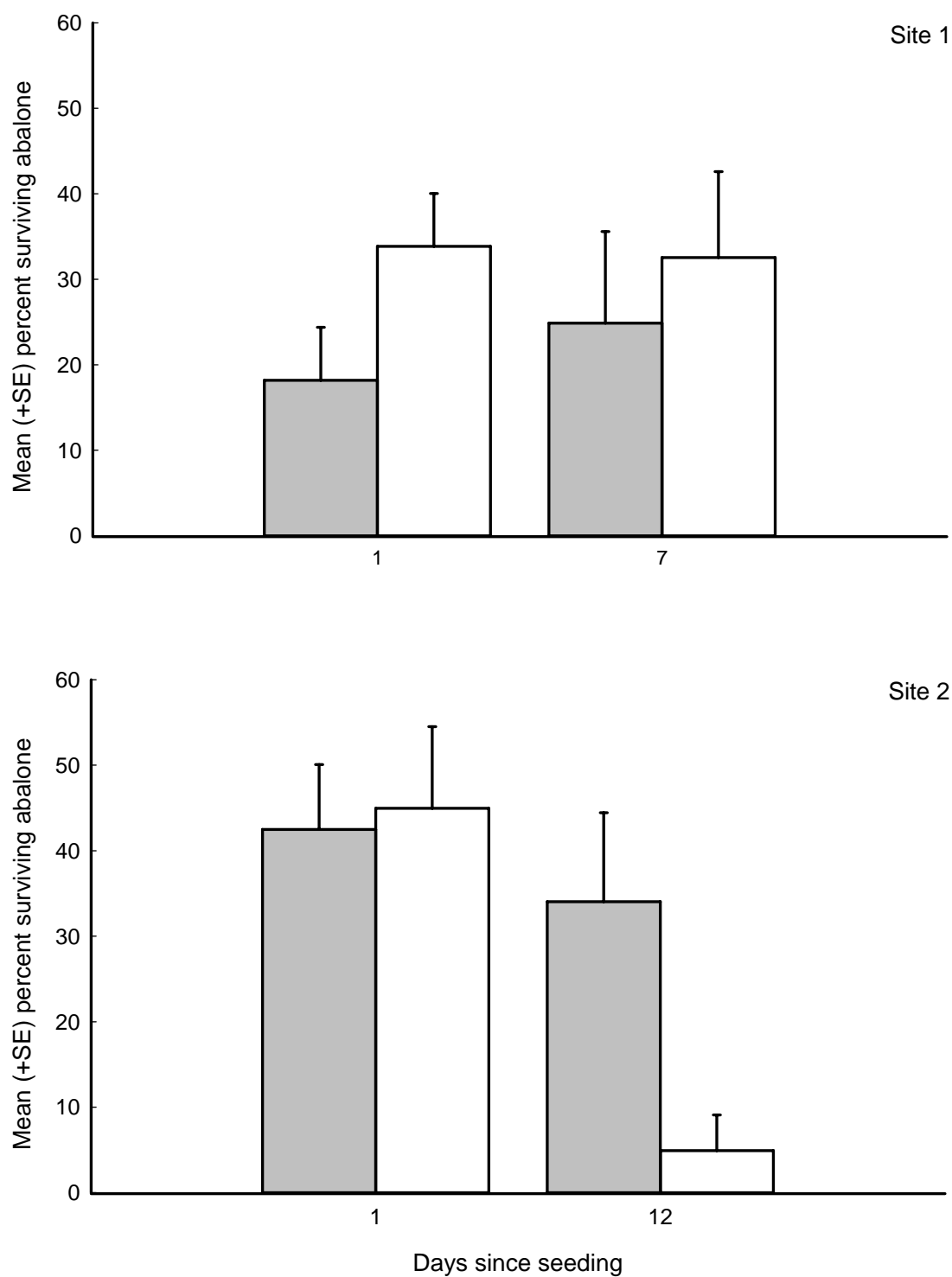
Figure 9. Mean (+S.E.; $n = 12$) percentage of abalone found per treatment which were still associated with an urchin, after seeding under (open bars) vs. near (shaded bars) urchins at two sites at Port Stephens.
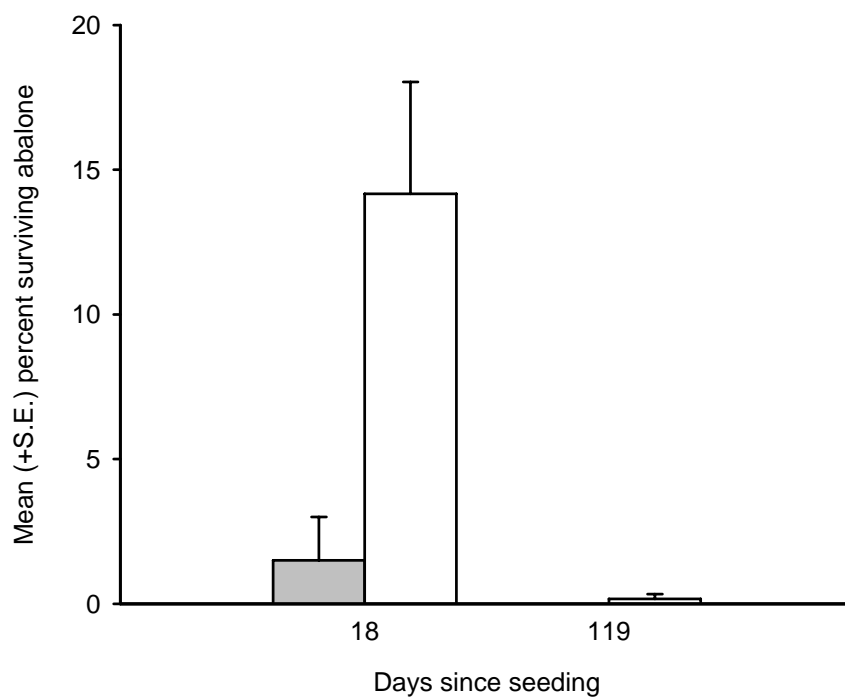
Figure 10. Mean percent (+S.E.; *n* = 5) of the total seeded abalone fed on a natural diet (red shell colour; shaded bars) and an artificial diet (turquoise shell colour; open bars) found at Port Stephens.
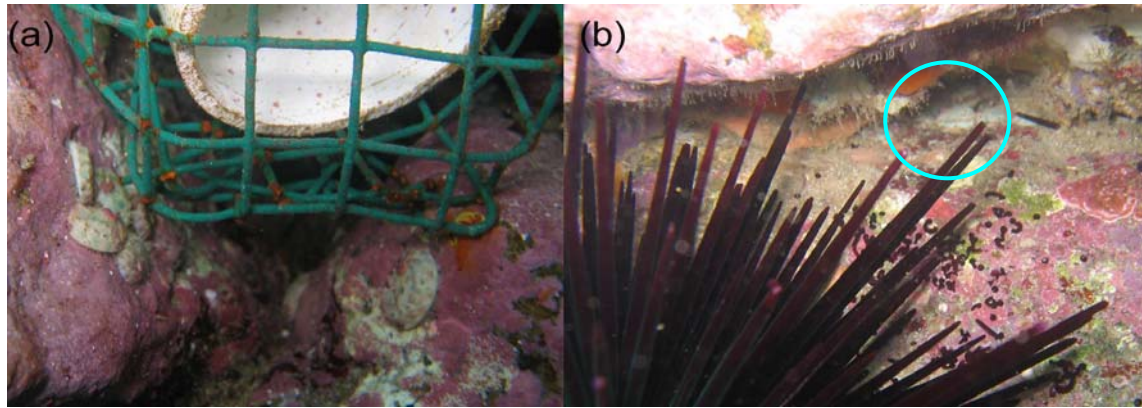
Figure 11. Hatchery-reared juveniles found after 1 month aggregated in cracks and crevices (microhabitat) near (a) the seeding devices and (b) the urchin *C. rodgersii* (observe the turquoise shells inside the blue circle). Note that in (b) the urchin was moved from the crevice to sample, but was initially in the crevice.

4.2.2.   Evaluation of factors affecting post-release survival of hatchery reared juveniles: Low density

Experimental assessment of the survival of cultured *Haliotis rubra* seeded over extensive areas of juvenile habitat at Port Stephens at very low densities typical of wild populations under natural recruitment – <u>Progress report only</u>

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4.2.2.1.   Introduction and background

A simple bio-economic model (see Section 4.4) for enhancing depleted populations of *H. rubra* was used to compare the benefits and costs of five alternative age/size classes of hatchery produced seed. The seed ranged from week old competent larvae to 18 month old 40 mm juveniles. Age specific natural mortality rates and seed production costs were used to predict and compare potential net income of seeding the 5 size classes. "Button size" (7 – 15 mm SL 6 – 9 months) juvenile seed were identified as having the best prospects for achieving cost-effective enhancement.

Outcomes of an earlier project (FRDC 98/219, Heasman *et al.* 2004) that included a comprehensive array of seeding experiments using "button size" juveniles had been generally disappointing. Almost a million juvenile *H. rubra* (mainly in the range of 5 to 15 mm) were deployed in large clusters of 700 to 2,500 within predator protective release devices at more than 40 sites from Port Stephens to the Victoria/NSW border. While average growth rates were similar at all sites and similar to that of wild stock, survival after a year averaged only 0 – 4% and compared poorly with documented rates of 12 – 40% for their wild counterparts.

These low survival rates prompted a review of published information on the natural biology of juvenile *H. rubra*. This revealed that natural densities of 12 – 18 month old juveniles on natural rocky reef habitats were consistently low, commonly 2 or less per square metre of reef. This knowledge prompted a final seeding experiment within FRDC project 98/219 to test the hypothesis that "button size" juveniles should be seeded sparsely to overcome much higher than normal losses thus far experienced (Heasman *et al.* 2004). Results one year after release supported this hypothesis. Average survival of "button size" juveniles (7 – 15 mm) seeded at each 115 m^2 site ($\sim$ 9/m^2) as 10 dispersed groups of 100 was 12.5%. This was within the expected normal range of wild abalone and more than 3 times the average rate of 3.8% for abalone seeded in single clusters of 1,000. This result suggested that continuation of this line of research was warranted but raised the need to develop new simple, cheap, versatile and reliable methods of achieving a low density dispersal of seed over large areas of juvenile habitat.

The factor driving this line of research towards development of cost-effective enhancement of *H. rubra* fisheries was the continuing general decline in *H. rubra* stocks throughout NSW. This decline has been principally attributed to overfishing by the commercial sector combined with increasing pressure from recreational fishers and illegal poaching. Regardless of the cause, the continued decline prompted a further drastic cut in the total allowable commercial catch quota from

218 tonnes in 2004 to 130 tonnes in 2005. Compounding these problems, outbreaks of the disease *Perkinsus* between 2000 and 2002 decimated *H. rubra* stocks north from Jervis Bay to Port Stephens, with stocks around Port Stephens suffering estimated losses of more than 90%.

4.2.2.2. Aims

This program of research aims to:

1. Develop low density dispersal protocols that will enable large areas of depleted *H. rubra* stocks to be seeded using hatchery produced stock.
2. Assess short and longer term ecological impacts of seeding enhancement especially on type and abundance of macrophytes and the impact of major competitive species (especially other large grazing gastropods and urchins) on seed survival.
3. Assess genetic impacts over time and space of large-scale dispersed seeding of depleted wild fisheries stocks with large numbers of hatchery produced seed generated from small numbers of parent stock.
4. Assess long term yields and cost-effectiveness of large-scale seeding of depleted wild fisheries stocks with large numbers of hatchery produced seed.

4.2.2.3. Materials, methods, results and conclusions

(1) Design and operation of dispersed seeding release devices

Development of a reliable cost-effective method for low density dispersal of "button size" juveniles involved the design and manufacture of small predator protective release devices (capsules) (Figure 1). These are made of 16 x 16 mm square aluminium tubing (wall thickness of 1.5 mm). The tubing is cut into standard lengths that span the 332 x 332 mm floor of seedling crates (Nally Plastics P/L; model IH012). The seeding crates (Figure 2) comprise an open grid base and solid walls. Nineteen 332 mm lengths of tube can be packed into the bottom of the crates thereby creating a very smooth and level false floor (Figure 3).

Six release devices comprising 55 mm sub-lengths of tube are created by applying five equidistant deep saw cuts in the 332 mm lengths of tube. The six sub-lengths are linked together by small slivers of uncut metal in the bottom wall. Prior to inserting the deep cuts, six equally spaced 12 mm holes are drilled at the centre of the upper side of each 55 mm sub-lengths to provide access portals by which juvenile abalone are able to freely migrate to and from the lumen of the tubes. Lines of smaller 1.5 mm drainage holes are drilled in the lower wall of aluminium tube opposite the entry/exit portals. The latter allow free passage of seawater.

Seed abalone are anaesthetised for 10 minutes in a 1 mg/L solution of benzocaine in seawater at 18 – 20°C before being evenly dispersed over the false floors created by the release devices (Figure 3). A random sample of juveniles is used to determine average numbers per 100 g drained live weight. A subsample of 100 to 200 of these is preserved in 70% ethanol for subsequent determination of mean shell length and the size frequency distribution. The anaesthetised juveniles are regularly observed for at least 2 hours, a period that embraces an initial post-anaesthesia recovery of about 20 minutes. The common initial behavior of the recovering juveniles is to form patchy clusters that persist for up to an hour or so. Generally 95 to 99% of abalone will migrate into the lumen of the devices within 1 to 3 hours and remain there for several days under the influence of intense light (Figure 4).

In preliminary trials it was determined that each capsule could accommodate 4 to 5 g of juveniles in mixed size, nursery reared batches in the range 5 – 18 mm. This translates to an average of about 18 juveniles, averaging 11 mm and 0.25 g per capsule (total displacement volume of juveniles per

capsule is about 3 ml). The upper limit on size imposed on juveniles entering was determined by shell width (see Figure 4). This approximated two thirds of the corresponding shell length of "button size" juveniles in the range 5 to 20 mm shell length (Figure 5).

Seedling crates loaded with stocked capsules are positioned level within troughs fitted with a standpipe overflow set at a height that provides about 50 mm of seawater above the upper surface of the devices. Fresh 18 – 21°C coastal seawater (salinity of 34 – 35 g/L) is continuously applied at a rate of 5 litres per minute. The troughs can be located outside if prevailing ambient temperatures are in the range 17 – 22°C, otherwise they can be housed in an insulated air conditioned room at 18°C. Intense lighting is provided by a 40 w fluorescent tube or 100 watt incandescent lights mounted 1 m above the troughs.

Once loaded into the capsules, the stock can be maintained in good health for up to several days awaiting favourable sea and weather conditions or protracted transportation to distant release sites. The capsules can be broadcasted onto suitable juvenile habitat areas of reef during daylight hours from surface craft, thereby negating prohibitive costs of manual seeding of such large areas by SCUBA.

Figure 1. Individual 55 x 16 x 16 mm aluminium release capsule. Note 12 mm entrance portal and 1.5 mm drain holes.
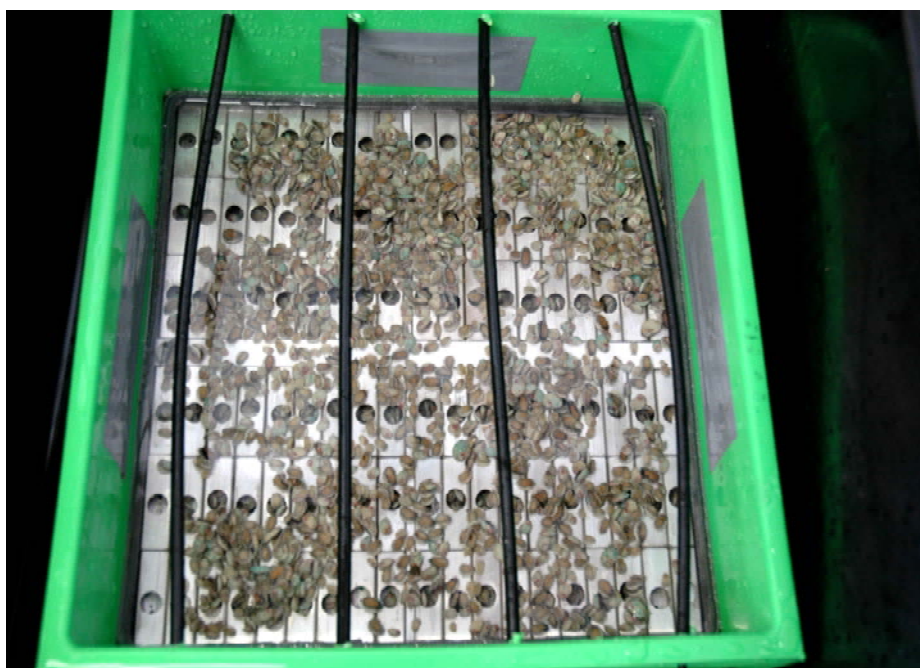
Figure 2. **Top:** Close-up of "button size" juveniles recovering from anaesthesia and self-loading into release devices via 12 mm portals. **Bottom:** Plastic tray stacked with single layer comprising 114 abalone seeding capsules. These have been stocked with a total of about 2000 "button size" juveniles still in the process of recovering from benzocaine induced anaesthesia and self-loading into the lumen of the capsules.

Figure 3. 335 x 325 mm, plastic trays (*Nalley* Aust. P/L) adapted to accommodate a single layer of release capsules carrying self loaded "button size" (mean 11 mm/0.25g) juvenile *H. rubra* awaiting transportation to seeding sites. Capsules accommodate an average of ~18 juveniles (~ 2000 juveniles/ tray). **Right:** Note high rate of aeration that is combined with a net seawater exchange rate equal to or exceeding the biomass of juveniles every minute to ensure stock remains in peak health and vigour. **Left:** Aeration turned off to reveal that > 98% of juveniles remain ensconced within the lumen when continuously exposed to intense light.
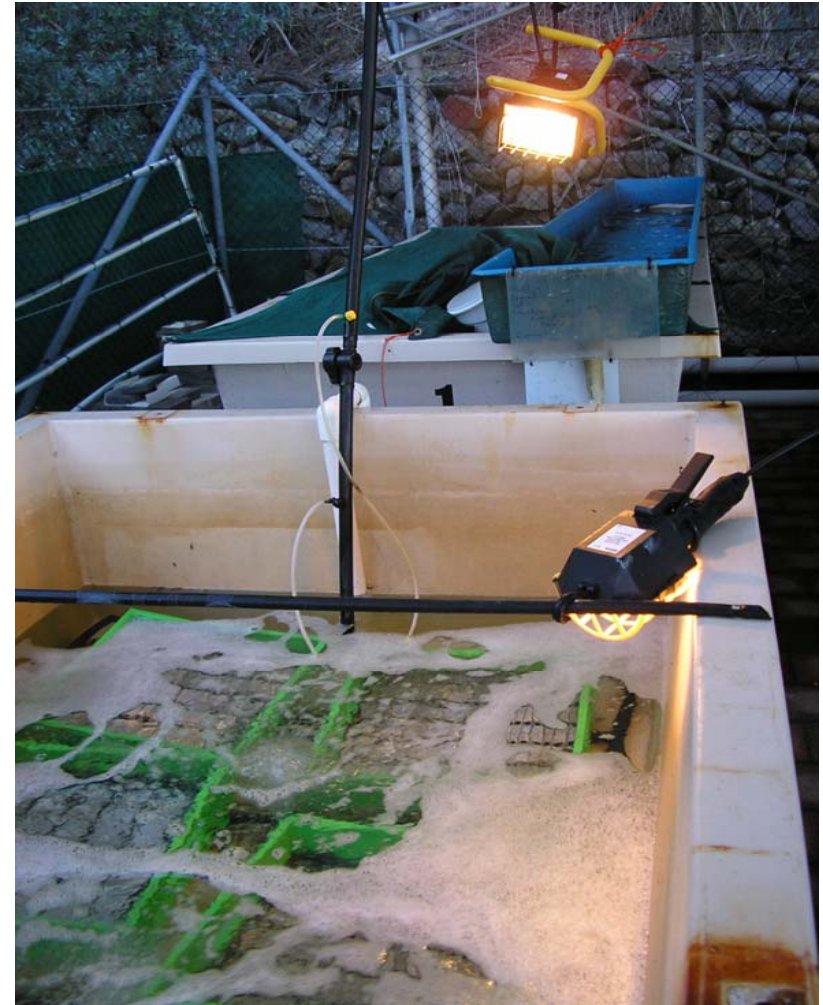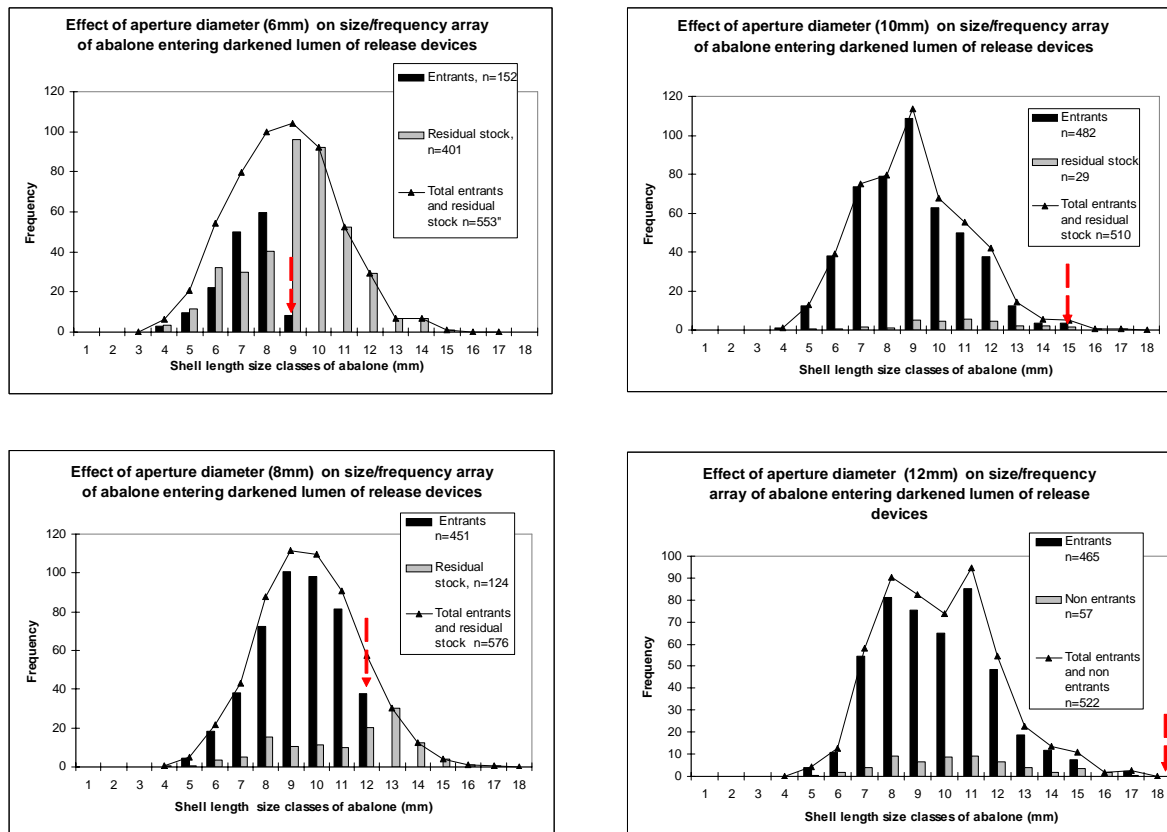
Figure 4. Effect of l diameter of capsule portal on size frequency array of juvenile *H. rubra* that enter and remain in the lumen of capsules in avoidance of strong incident light. Red arrows designate upper cut-off size of entrants. **Note:** Cut-off occurs where shell width = portal diameter.
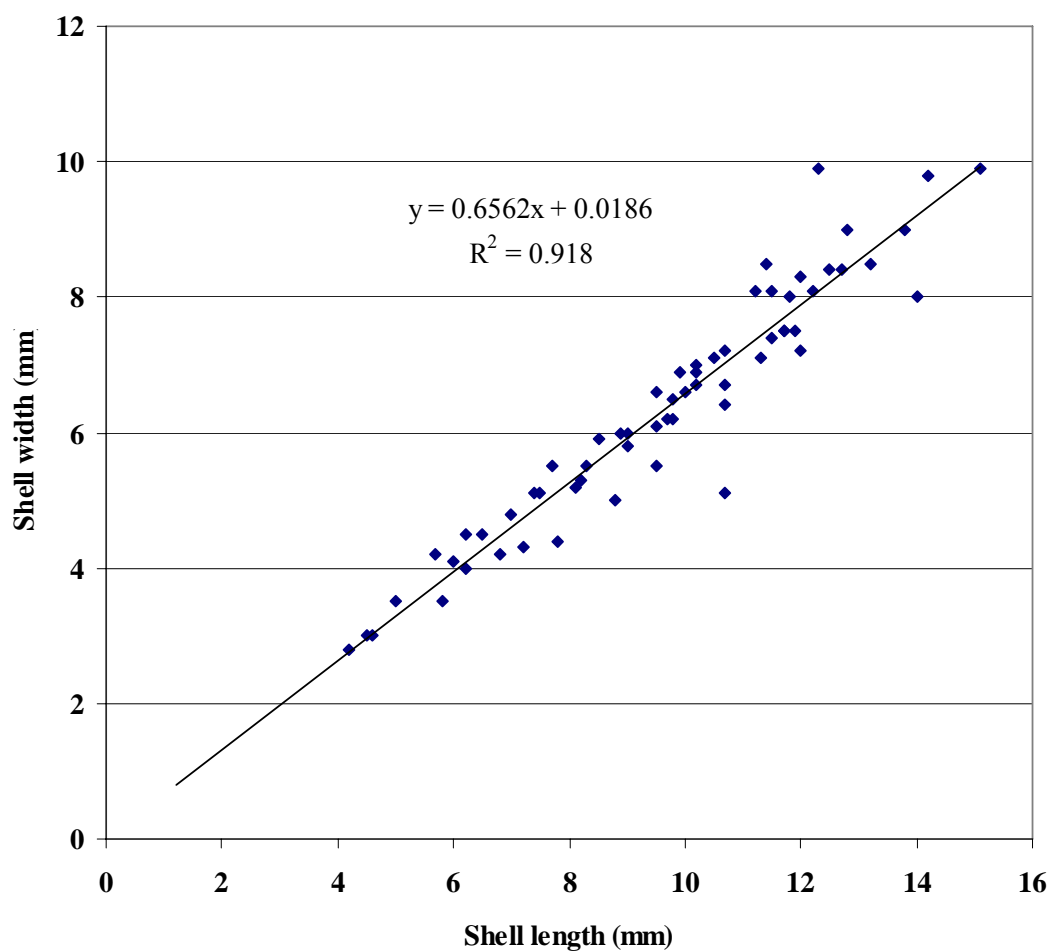
Figure 5.   Shell length vs width data for juvenile *H. rubra* in the range 4 to 15 mm

(2) *Assessment of short and longer term ecological impacts of large-scale dispersed seeding of depleted abalone populations*

Sites selection and seeding protocols

Twenty potential sites along the coast of Port Stephens (between Fingal Island and Birubi Point) were initially identified as likely candidates for this experiment after consultation with a total of five experienced commercial, recreational and research divers. After further pre-inspection by SCUBA, 12 of the 20 (Figure 6) were selected for the experiment. In all cases these sites were in areas where both juvenile and adults abalone were known to regularly occur. The sites contained suitable habitat for juvenile abalone (i.e., rock substrates with small cracks and fissures) and small boulder fields along with habitat that typically supports adults (i.e., crevices and larger boulder fields). Sites were selected far enough apart (≥30 m) to minimise the probability of migration of abalone between any adjacent sites (Hamer 1982).

Sites were approximately 1000 m^2. Six sites were randomly selected for seeding and the remaining six were unseeded (controls). During July and August 2005, juvenile abalone ranging in size from (7 to 14 mm) were deployed from the surface at each seeded site using small predator protective capsules described above. Seeding density was 8.6 to 10.1/m^2 (i.e., 8,592 to 10,098 juveniles per site) and was completed from a 10 m dive boat in 5 to 10 minutes per site. These abalone were distinguished from wild counterparts by a distinctive blue green shell colouration imparted by a formulated diet used in their hatchery production.

Survey protocols

Between August and December 2005, surveys were conducted at all sites to estimate the numbers of invertebrate grazers (large species that might compete with abalone such as the common urchin *Centrostephanus rodgersii* and other large surface grazing snails) and to estimate percentage cover of dominant habitats. Habitats (Figure 7) were classified in the following categories:

1. *Ecklonia radiata* (kelp)
2. *Phyllospora comosa* (large kelp-like brown alga)
3. *Sargassum spp.* (intermediate-sized brown algae)
4. *Cystophora spp.* (intermediate-sized brown algae)
5. Small brown foliose algae (e.g., *Zonaria* spp., *Dictyota* spp.)
6. Branching coralline algae (e.g., *Corallina officinalis*, *Amphiroa anceps*)
7. Pink encrusting coralline algae
8. Red encrusting algae (e.g., *Peysonellia* spp.)
9. Turfing algae (e.g., brown, red and green filamentous species)
10. *Caulerpa filiformis* (short green strap weed)
11. Sponge
12. Sand
13. Bare rock

Numbers of large surface grazers were estimated using six replicate 10 m long x 1 m wide transects placed haphazardly in sites. Divers recorded numbers of large grazers, i.e., purple urchins (*Centrostephanus rodgersii*), pink urchins (*Heliocidaris erythrogramma*), turban shells (*Turbo torquatus* and *Turbo militaris*), tent shells (*Astralium tentorium*) and adult abalone (*Haliotis rubra*), in each transect. Habitat cover was sampled using the same transects with divers recording the dominant habitat every 1 m along a 10 m tape measure.

Abundance estimates for wild (adult) abalone were made using timed searches in each site. These searches involved a single diver swimming within the site for 10 minutes and looking amongst boulders and in crevices, but no boulders were overturned. Any abalone found were collected,

measured (maximum shell length) and a small tissue biopsy was taken before they were returned to the site by divers.

Abundances of grazers were compared among sites using Analyses of Variance. When necessary, data were transformed to ln(x + 1) to make variances homogeneous. Multivariate analyses (PRIMER software package) using Bray Curtis similarity matrices were used to compare habitats among sites. For univariate and multivariate analyses, a two factor design was used, i.e., "Treatment" (seeded vs unseeded, fixed factor) and "Site" (random, nested within seeded or unseeded). Because adult abalone were not sampled in replicate transects, a simple one factor ANOVA was used to compare total abundances between seeded and unseeded treatments, with the six sites in each treatment being the replicates.

Approximately three years after the initial seeding, this sampling protocol will be repeated. In addition, searches for tagged abalone (calceine tag) will be done over larger areas at each site embracing adjacent and readily accessible adult habitat to account for movement of the seeded animals.

4.2.2.4. Results

Invertebrate grazers

The dominant grazer found at most sites was the large urchin *Centrostephanus rodgersii* (ranging from 0.2 – 4.2 per m^2), followed by the gastropods *Turbo torquatus* and *T. militaris* (0.2 – 1.3 per m^2). The small urchin *Heliocidaris erythrogramma* was rare at most sites (ranging from 0 – 0.4 per m^2).

Abundances of all invertebrate grazers differed significantly among individual sites, but no significant differences were detected between seeded and unseeded treatments (i.e., comparing the average of the six sites in each treatment, Table 1, Figure 8). Similarly, there was no significant difference in total numbers of abalone between seeded and unseeded treatments (Table 1), although there was considerable variability among replicate sites within each treatment (0 – 31 in seeded sites; 0 – 27 in unseeded sites) (Figure 9).

Sizes of wild adult abalone ranged from 74 – 135 mm at seeded sites and from 82 – 160 mm at unseeded sites. The size frequency of abalone for all 12 sites was roughly normally distributed around the minimum legal length of 115 mm (Figure 10).

Habitats

There was no significant difference in percentage covers of habitats between seeded and unseeded treatments (ANOSIM, $P > 0.7$), but there was significant variation among individual sites within each treatment (ANOSIM, $P < 0.01$). An MDS ordination was used to represent the habitats at each site (the closer the symbols in the plot, the more similar the habitats). This ordination confirmed the ANOSIM results, with no clear grouping of seeded and unseeded sites and large separation of individual sites (Figure 10). The small stress value for this plot means that the ordination is a very good two dimensional representation of the habitat data.

4.2.2.5. Conclusions

Subtidal habitats and numbers of macro-invertebrates were similar in seeded and unseeded treatments. The large variability among sites within each treatment was expected and is one reason why large numbers of sites are needed. If the seeding of juvenile abalone has any effect on total numbers of abalone or habitats, then we should see the seeded sites becoming more similar over time as the unseeded sites continue to be different.

Table 1.   Summary of ANOVA results comparing abundances of invertebrates grazers between Seeded and Unseeded treatments (1 and 10 degrees of freedom) and among sites (10 and 60 degrees of freedom) within each treatment; ns = not significant ($P > 0.05$).

Species	Transform	Treatment	Site
C. rodgersii	ln(x+1)	ns	*P* < 0.001
H. erythrogramma	none	ns	*P* < 0.05
T. torquatus and T. militaris	ln(x+1)	ns	*P* < 0.05
A. tentorium	none	ns	*P* < 0.001
H. rubra	none	ns	No test
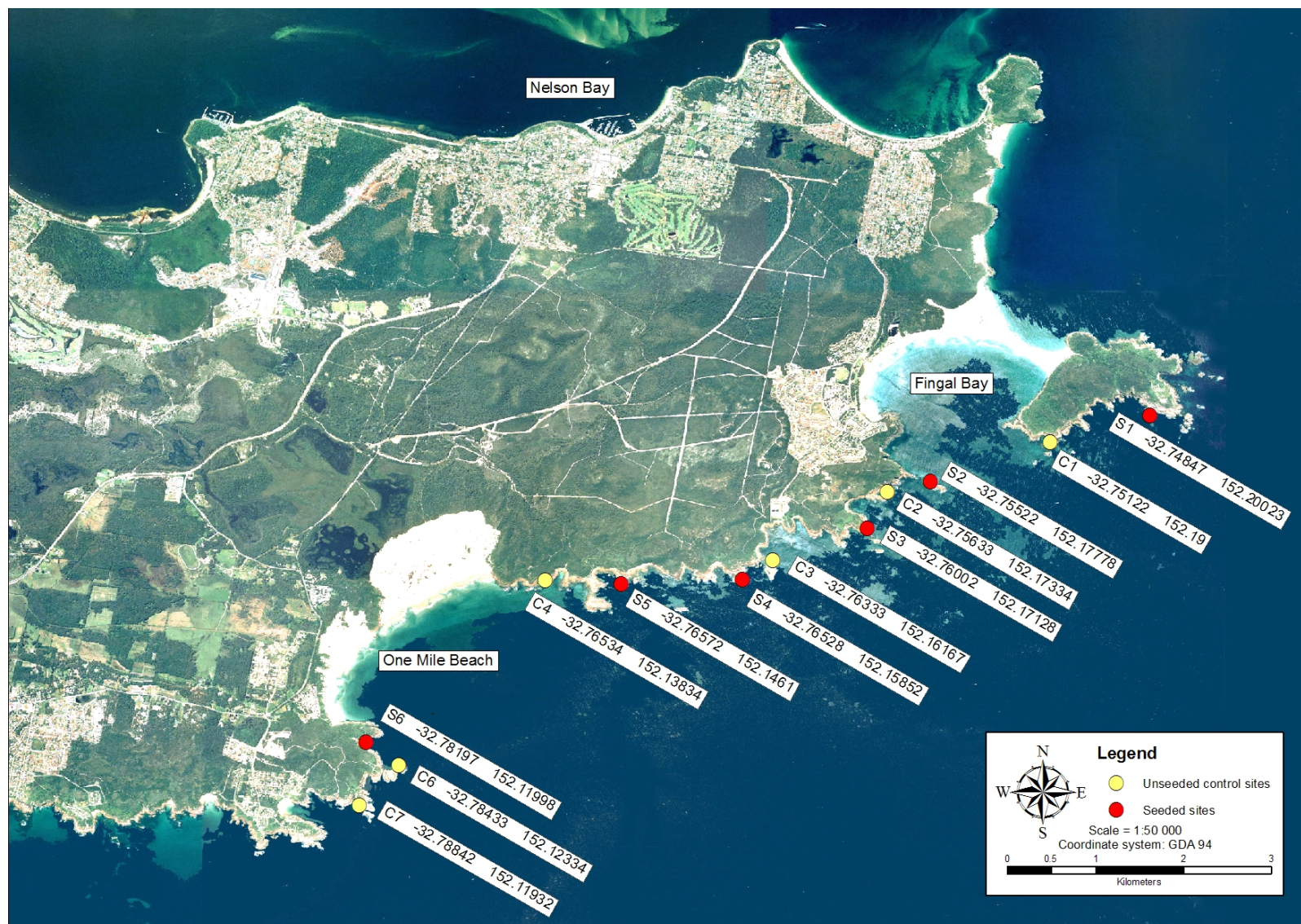
Figure 6. Locations (with latitudes and longitudes) of the six seeded and six unseeded sites along the southern coastline of Port Stephens.
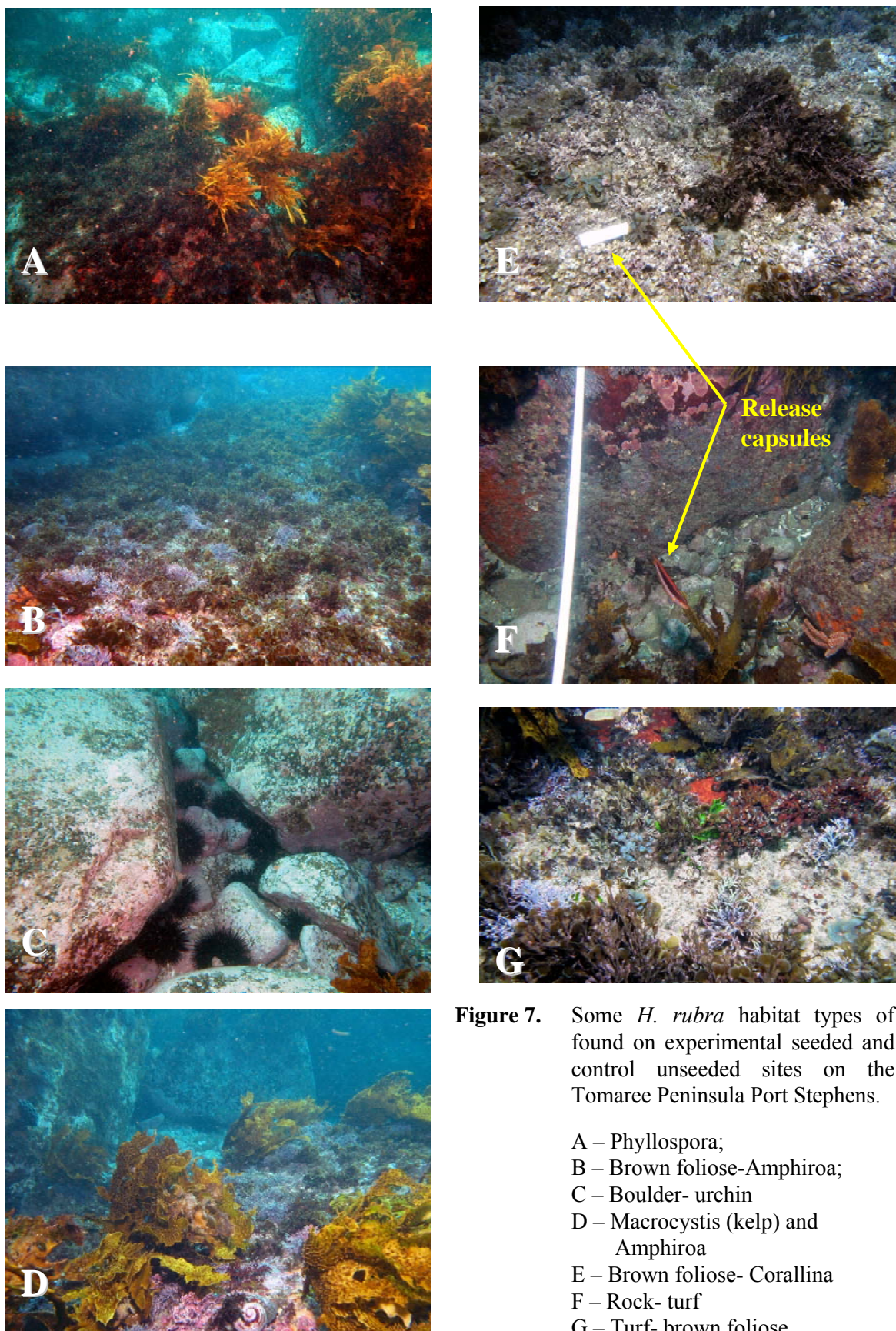
Figure 7. Some *H. rubra* habitat types of found on experimental seeded and control unseeded sites on the Tomaree Peninsula Port Stephens.

A – Phyllospora;
B – Brown foliose-Amphiroa;
C – Boulder- urchin
D – Macrocystis (kelp) and
    Amphiroa
E – Brown foliose- Corallina
F – Rock- turf
G – Turf- brown foliose
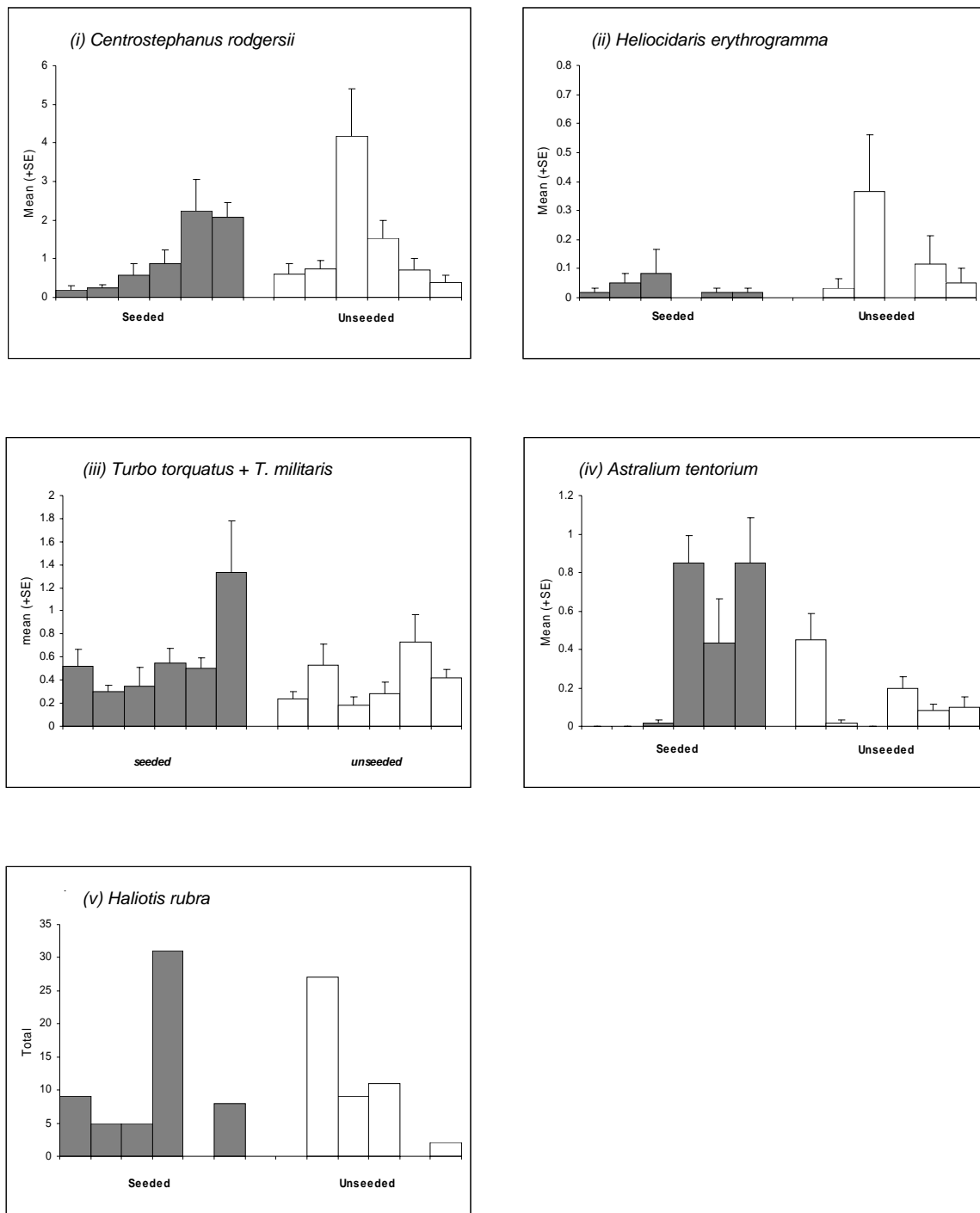
Figure 8. Mean numbers (+SE) of grazers per square metre at each seeded and unseeded site. (i)
Centrostephanus rodgersii, (ii) *Heloicidaris erythrogramma*, (iii) *Turbo torquatus* and
T. militaris, (iv) *Astralium tentorium* and (v) total number of *Haliotis rubra*.
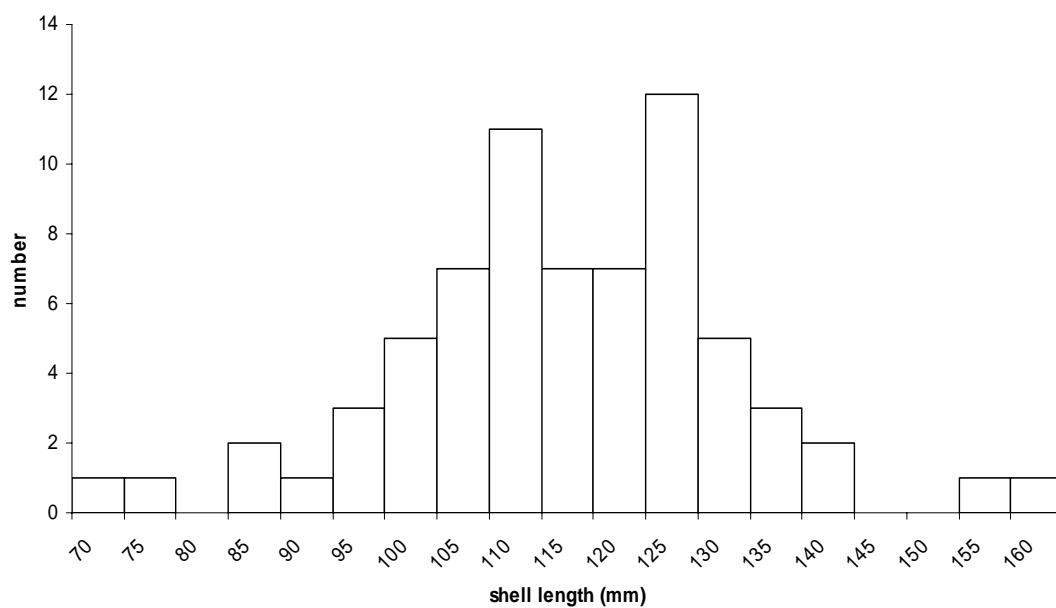
Figure 9. Size frequency (maximum shell length) of wild abalone from all 12 sites (seeded and unseeded combined).
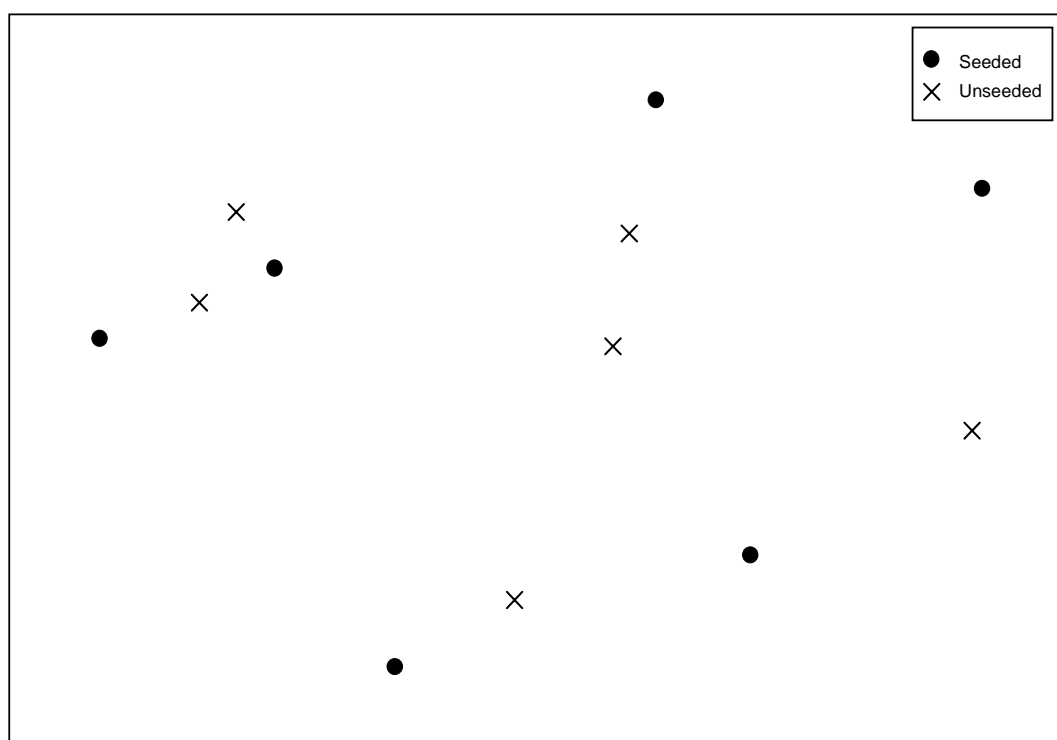
Figure 10. MDS ordination for comparing habitats among six seeded (dots) and six unseeded (crosses) sites. Stress value for two dimensional ordination is 0.08.

(3)   Assessment of short and longer term genetic impacts of large-scale dispersed seeding of depleted abalone populations (prepared by David Hurwood, QUT)

Background

With respect to population genetics, there are several issues relating to possible impacts of seeding operations on natural populations of abalone (as with other potential marine culture species). These issues can be broadly formalised into two goals: a) optimising genetic diversity in culture lines, and b) conserving local wild gene pools.

Prior to large scale seeding programs being undertaken, collection of preliminary genetic data can help to address these issues. Of primary interest is how much diversity exists in the wild and how is it distributed spatially (i.e., the population or stock structure). We are interested in stock structure because it would be unwise to seed populations with genetically divergent hatchery stock. If wild stocks interbred with genetically divergent individuals, there is the potential for outbreeding depression that could reduce the overall viability of the wild gene pool.

Concurrent with identification of wild stock structure is the assessment of genetic diversity. Once this has been established, we can make informed decisions regarding broodstock selection and breeding protocols. Monitoring genetic diversity over generations within hatcheries can also identify any reductions in genetic diversity due to inbreeding and thereby design hatchery strategies to prevent the potential loss of productivity through inbreeding depression.

Genetic attributes of the seeding operation are factors that should be considered at the beginning of the process. The information collected at this stage will also provide some insight for long term issues. These relate to how seeding abalone change the genetic makeup of local populations where seeding has been undertaken and also importantly, the success of the seeding program itself (i.e., how has seeding contributed to the harvestable population).

This section presents the genetic data collected as a preliminary assessment of wild genetic diversity in abalone stocks around Fingal Heads where the seeding experiments were performed.

Methods

– Determining levels of genetic variation in wild and cultured populations of H. rubra

Primers for a GelScan 2000 (Corbett) system using tissue biopsies from wild caught *H. rubra* were optimised. Twelve microsatellite primer pairs were initially assayed using tissue from 30wild caught individuals from Broughton Island for their application in determining the level of genetic variation in populations of *H. rubra*. From these, six microsatellite loci (see Table 2) were established to assay levels of genetic variation for further wild populations of *H. rubra*).

A total of 100 wild adult *H. rubra* collected by SCUBA at three adjacent locations approximately 200 – 300 metres apart around Fingal Head, Port Stephens, were assayed for the six selected polymorphic loci. Number of alleles detected for each of six loci in tissue samples from each of the three locations around Fingal Head are provided in Table 3. The genotype for five broodstock (see Table 4) contributing to a batch of juveniles produced at the NSW DPI Shoal Bay hatchery for use in the mass seeding experiment was also determined.

Table 2.   Loci selected for the study. [1]Microsatellites isolated and primers designed by Evans *et al*. (2000). [2]Microsatellite isolated and primers designed by Huang and Hanna (1998).

Locus	Repeat Motif	Allele Size Range	No. of Alleles
Hr1.24[1]	$(AT)_8$	221 – 227	4
Hr2.36[1]	$(AC)_{21}$	87 – 131	15
Hr2.9[1]	$(GT)_{27}$	178 – 258	20
RubCA[2]	$(CA)_{12}$	134 – 186	18
Hr1.14[1]	$(GT)_{13}TT(GT)_2GA(GT)_3$	252 – 262	3
Hr2.14[1]	$(GAGT)_8…(GAGT)_5$	208 – 258	9

Table 3.   Samples from three locations at Fingal Head assayed for six loci. The three tables listed below (a – c) display the number of alleles detected for each locus from each of the three sites. These values represent a preliminary assessment of variation in the wild (i.e., the number of alleles and the allelic range).

a)   Location 1 sample

Locus	Sample Size	Allele Size Range	No. of Alleles
Hr1.24	27	221 – 225	3
Hr2.36	16	95 – 131	11
Hr2.9	16	176 – 240	18
RubCA	22	136 – 178	16
Hr1.14	27	252 – 262	3
Hr2.14	26	208 – 254	8

b)   Location 2 sample

Locus	Sample Size	Allele Size Range	No. of Alleles
Hr1.24	23	221 – 225	3
Hr2.36	17	91 – 119	7
Hr2.9	9	182 – 234	9
RubCA	n/a	n/a	n/a
Hr1.14	23	252 – 262	4
Hr2.14	22	212 – 236	6

c)   Location 3 sample

Locus	Sample Size	Allele Size Range	No. of Alleles
Hr1.24	n/a	n/a	n/a
Hr2.36	20	95 – 117	10
Hr2.9	15	176 – 222	13
RubCA	30	130 – 178	17
Hr1.14	40	252 – 262	3
Hr2.14	29	200 – 236	8

Table 4.   Genotypes of five individuals (3 male and 2 female) representing the broodstock for rearing larvae/juveniles for release into the wild assayed for variation using six loci.

Sample	Sex	Hr1.24		Hr2.36		Hr2.9		RubCA		Hr1.14		Hr2.14	
		1	2	1	2	1	2	1	2	1	2	1	2
1914	M	221	221	109	109	184	190	na	na	260	262	224	224
1757	M	221	221	99	99	190	222	170	170	260	260	224	228
1248	M	221	221	93	93	na	na	160	160	260	262	224	224
1915	F	221	221	99	99	198	212	154	166	252	260	224	236
1752	F	221	223	95	95	190	230	162	166	260	260	224	236

– Statistical methods employed to examine variation at six microsatellite loci among wild and cultured samples of H. rubra.

A total of 69 individuals from nine seeding sites were sampled with sample sizes (n) given in Table 5. Also, samples of 40 individuals each from two hatchery lines (one from 'raceway' and one from 'settlement tank' (S2-1)) were included to investigate hypothesised reductions in genetic variation due to inbreeding.

Table 5.   Sampling dates and sample sizes of nine prospective seeding sites and two hatchery lines prior to release of hatchery stock.

Date sampled	Site/Label	Sample size (n)
9/8/05	12A	9
9/8/05	6A	5
9/8/05	4B	5
2/11/05	3A	10
2/11/05	C5	9
2/11/05	10	10
17/1/06	2	11
17/1/06	14A	2
24/1/06	11B	8
Total Wild		*69*
	R1	40
	S2	40
Total Hatchery		*80*

Preliminary analyses were undertaken to determine whether the microsatellite loci chosen were appropriate for addressing the specific goals of the project. Firstly, the data were tested for linkage disequilibrium using the software package ARLEQUIN version 3.0 (Excoffier *et al.* 2005) to ensure that each locus was providing an independent assessment of genetic variation among samples. Linkage disequilibrium (LD) can arise by several means. If two loci are situated closely on the same chromosome, then they will not assort randomly from one generation to the next. If two loci are linked in this manner, then a significant measure of LD will be detected for every sample. If, on the other hand, significant measures of LD arise at different loci pairings for

different loci, then the cause is more likely to be the result of biological processes (e.g., unequal contribution of broodstock to the next generation) or a sampling effect.

Other preliminary tests carried out were to determine the likelihood of error arising in the scoring of electrophoresis gels (i.e., genotyping errors). Common errors include scoring stutter bands as true alleles, small allele dominance (where smaller alleles are preferentially amplified during PCR) and the presence of null alleles (where alleles fail to amplify due to a mutation at the priming site). The first source of error may increase levels of variation, whereas the other two will result in an underestimate of true variation. The possibility of genotyping error was investigated using MICROCHECKER (Van Oosterhout, 2004) and where null alleles are suspected, null frequencies were estimated by a number of methods (Brookfield, 1996; Chakraborty *et al.,* 1992; Van Oosterhout, 2006).

Seven different methods for assaying genetic variation were used in order to 1) determine whether there is a significant reduction in genetic variation among seeding sites and hatchery F1 stocks, and 2) to evaluate which measure/s of variation are the most appropriate and/or sensitive to detecting differences among samples. The methods were:

i) The number of alleles per locus.
ii) The allelic range (difference between the smallest and largest allele).
iii) Observed heterozygosity (Ho).
iv) Expected heterozygosity (under Hardy-Weinberg expectations)
v) Allelic richness (El Mousadik and Petit, 1996) based on the principle of estimating the expected number of alleles in a sub-sample of 2n genes, given that 2N genes have been sampled.
vi) Genetic diversity (Nei, 1987) which is the probability that two randomly chosen alleles are different in the sample.
vii) Theta (H) (or sometimes θ_{hom}) is the estimate of θ ($4N\mu$) based on expected homozygosity in a population at equilibrium between drift and mutation.

Methods i-iv and vii were estimated using ARLEQUIN. The remainder were estimated using FSTAT version 2.9.3.2 (Goudet, 2002).

Results and Discussion

Results for the LD tests are provided in Table 6(a-k). While some pairwise comparisons are significant ($P < 0.05$), there is no common pattern across samples indicating that there is not a problem with linkage and that all loci are independent measures of diversity.

Tests for genotyping errors revealed no evidence for small allele dominance. Furthermore there was little evidence for falsely scoring stutter bands. The analysis suggested the possibility of stuttering on three occasions (Locus Hr2.14 for sample 10-1 and Locus 2.36 for samples R1-1 and S2-1). These gels were re-examined with the original scoring confidently retained.

The presence of null alleles however, appears to be more problematic (Table 7). In one case, estimates of the frequency of null alleles in the sample exceeded 70% (for RubCA at 12A1) and many estimates were commonly over 20%. As a result, interpretation of levels of genetic diversity need to be undertaken cautiously, especially for loci RubCA, Hr2.9 and Hr2.36. It is interesting to note that while the hypothesised null allele/s for RubCA was found in many of the wild samples, it appears not to be present in the individuals selected for broodstock as there is no indication of a problem with null alleles in either hatchery sample (R1-1 or S2-1). However, the other two loci displaying a high frequency of null alleles appear to be in relatively high frequency in the broodstock.

Table 6.   Results of linkage disequilibrium analysis for each sample.
                   + indicates a significant association among two loci ($P < 0.05$)
                   ++ indicates a significant association among two loci after correction for multiple tests ($\alpha = 0.05/15$).

12A1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	++	-	*			
Hr2.9	+	-	++	*		
Hr1.24	+	-	+	-	*	
Hr2.36	-	-	+	-	-	*
6A1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	-	-	*		
Hr1.24	-	-	-	-	*	
Hr2.36	-	-	-	-	+	*
4B1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	-	-	*		
Hr1.24	-	-	-	-	*	
Hr2.36	-	-	+	+	-	*
3A1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	-	++	*		
Hr1.24	-	-	+	-	*	
Hr2.36	-	-	++	++	-	*
C5-1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	-	-	*		
Hr1.24	-	-	-	-	*	
Hr2.36	-	-	-	+	-	*
10-1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	+	+	*		
Hr1.24	-	-	-	+	*	
Hr2.36	-	+	+	-	-	*

Table 6.   Continued

2-1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	+	+	*		
Hr1.24	-	-	-	-	*	
Hr2.36	-	+	+	+	-	*
14A1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	-	-	*		
Hr1.24	-	-	-	-	*	
Hr2.36	-	-	-	-	-	*
11B1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	-	-	*		
Hr1.24	-	-	-	-	*	
Hr2.36	-	-	-	+	-	*
R1-1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	+	-	+	*		
Hr1.24	+	-	-	+	*	
Hr2.36	+	-	-	-	-	*
S2-1	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
Hr1.14	*					
Hr2.14	-	*				
RubCA	-	-	*			
Hr2.9	-	+	+	*		
Hr1.24	-	-	-	+	*	
Hr2.36	+	-	-	+	-	*

Table 7.   Estimated frequency of null alleles by locus for each sample. The range of estimated frequencies result from the use of four different methods of estimating null frequencies (Brookfield, 1996, Chakraborty *et al.*, 1992; Van Oosterhout 2006).

LOCUS

SITE	Hr1.14	Hr2.14	RubCA	Hr2.9	Hr1.24	Hr2.36
12A1	-	-	44 – 72%	-	-	-
6A1	-	-	-	-	-	-
4B1	n/a	n/a	n/a	n/a	n/a	n/a
3A1	-	-	15 – 44%	13 – 30%	-	12 – 29%
C5-1	-	-	-	-	-	22 – 38%
10-1	-	19 – 35%	18 – 42%	-	-	32 – 61%
2-1	-	-	-	-	-	-
14A	n/a	n/a	n/a	n/a	n/a	n/a
11B1	-	-	19 – 26%	26 – 55%	-	-
R1-1	-	-	-	10 – 20%	-	23 – 67%
S2-1	-	-	-	20 – 34%	-	21 – 43%
Total	**-**	**5 – 11%**	**17 – 33%**	**17 – 28%**	**-**	**26 – 51%**

– Measures of Genetic Diversity

Table 8 shows the seven different estimates of genetic variation for each locus at every sample. While there is much variation in the measures of genetic diversity among sites and loci presented here that constrains statistical rigour, there is an obvious trend among the data. Regardless of the locus and site in general, genetic diversity appears to be lower in the two hatchery stocks than in the wild. This is not surprising given that only five individuals in total were used as broodstock for the hatchery lines.

Interestingly, this tendency is reversed for locus Hr1.14 (Table 8a) where most of the measures were higher for R1-1 and S2-1 than the wild samples. The only measure that does not follow the trend is Theta (H) where R1-1 and S2-1 both tend towards the lower values. While the pattern is suggestive of a sensitivity of this measure to reduced genetic diversity, the reverse pattern is seen for locus Hr1.24, where all measures are lower for hatchery stocks except for Theta (H). It should be noted that both of these loci have relatively few alleles at any one site or in total, and therefore the effects of sampling error can be greatly magnified. This estimate of genetic diversity is therefore probably not the most informative where there is either low sample size, low allele number or both. Indeed, Zouros (1979) demonstrated that Theta (H) is likely to be an overestimate of genetic variation when using only one or a few loci.

Due to the variation in sample size, the measure for number of alleles can be misleading. Naturally, the fewer the number of individuals in the sample, the smaller the potential number of alleles. This can clearly be seen at the most allele rich locus (Hr2.9, see Table 8d) where sample 14A, while having 100% heterozygosity, has only two individuals and hence can only have a maximum of four alleles. For a similar reason, allelic range may not be a good indicator of genetic variation. Not surprisingly, there is a very strong positive correlation between number of alleles and allelic range ($R^2 = 0.757$, $P < 0.05$).

Table 8. Measures of genetic variation for all samples by locus. * indicate where expected heterozygosity (He) is significantly different from observed heterozygosity (Ho).

a)　　Hr1.14

Site	# Alleles	Allelic Range	Ho	He	Allelic Richness	Gene Diversity	Theta (H)
12A1	3	5	0.14286	0.38462	1.385	0.405	1.61250
6A1	2	1	0.40000	0.35556	1.356	0.350	1.68211
4B1	3	5	0.60000	0.51111	1.511	0.500	1.50099
3A1	2	1	0.12500	0.12500	1.125	0.125	4.07143
C5-1	3	6	0.25000	0.24167	1.242	0.241	2.22831
10-1	4	6	0.36364	0.39827	1.398	0.400	1.58637
2-1	2	1	0.20000	0.18947	1.189	0.189	2.75577
14A1	1	0	-	-	1.000	N/A	N/A
11B1	1	0	-	-	1.000	N/A	N/A
R1-1	3	5	0.62162	0.50019	1.500	0.498	1.50000
S1-2	3	5	0.57500	0.55285*	1.553	0.553	1.52260
Mean	2.45	3.182	0.36423	0.36208	1.296	0.362	2.05112
S.D.	0.934	2.522	0.19805	0.15001	0.198	0.149	0.81920

b)　　Hr2.14

Site	# Alleles	Allelic Range	Ho	He	Allelic Richness	Gene Diversity	Theta (H)
12A1	6	10	0.75000	0.77500	1.775	0.777	2.36738
6A1	3	3	0.60000	0.71111	1.711	0.725	1.93389
4B1	6	6	1.00000	0.84444	1.844	0.825	3.30639
3A1	5	5	0.77778	0.70588	1.706	0.701	1.90833
C5-1	5	5	0.66667	0.75163	1.752	0.757	2.17838
10-1	4	5	0.27273	0.59307*	1.593	0.609	1.57179
2-1	5	5	0.55556	0.70588	1.706	0.715	1.90833
14A1	2	1	1.00000	0.66667	1.500	0.500	1.75000
11B1	4	6	0.50000	0.60000	1.725	0.732	1.58333
R1-1	2	3	0.43590	0.48518	1.485	0.486	1.50176
S1-2	3	3	0.47500	0.44272	1.443	0.442	1.50176
Mean	4.091	4.727	0.63942	0.66196	1.658	0.661	1.95784
S.D.	1.379	2.219	0.22871	0.12609	0.132	0.131	0.50153

Table 8. Continued

c) RubCA

Site	# Alleles	Allelic Range	Ho	He	Allelic Richness	Gene Diversity	Theta (H)
12A1	5	14	0.00000	0.84848*	1.848	0.933	3.38929
6A1	9	25	0.80000	0.97778	1.978	1.000	22.51136
4B1	6	16	0.60000	0.91111*	1.911	0.950	5.67378
3A1	9	23	0.57143	0.91209*	1.912	0.940	5.73569
C5-1	9	22	0.75000	0.92500	1.925	0.938	6.70721
10-1	12	24	0.55556	0.95425*	1.954	0.979	10.95254
2-1	6	10	0.33333	0.76471*	1.765	0.792	2.27885
14A1	1	0	-	-	1.000	N/A	N/A
11B1	9	17	0.50000	0.90833*	1.908	0.938	5.50500
R1-1	4	6	0.25000	0.63741*	1.629	0.629	1.64195
S1-2	4	6	0.52632	0.61544	1.615	0.617	1.61261
Mean	6.727	14.818	0.48866	0.83940	1.767	0.871	6.60083
S.D.	3.018	8.043	0.23813	0.19574	0.283	0.141	5.94448

d) Hr2.9

Site	# Alleles	Allelic Range	Ho	He	Allelic Richness	Gene Diversity	Theta (H)
12A1	8	28	0.71429	0.85714	1.857	0.869	3.58333
6A1	8	49	0.80000	0.93333	1.933	0.950	7.53571
4B1	6	25	0.50000	0.92857*	1.929	1.000	7.03846
3A1	10	40	0.62500	0.92500*	1.925	0.946	6.70721
C5-1	12	38	0.87500	0.95833	1.958	0.964	12.02174
10-1	12	33	0.72727	0.93939	1.939	0.950	8.28226
2-1	13	42	0.77778	0.96078*	1.961	0.972	12.77041
14A1	4	4	1.00000	1.00000	2.000	1.000	N/A
11B1	6	23	0.33333	0.86364*	1.864	0.917	3.74561
R1-1	5	20	0.52632	0.78351*	1.784	0.787	2.44772
S1-2	5	20	0.32500	0.66171	1.662	0.666	1.73364
Mean	8.091	29.273	0.654908	0.89194	1.892	0.911	6.58661
S.D.	3.059	12.159	0.215898	0.09695	0.097	0.102	3.59927

Table 8.   Continued

e)   Hr1.24

Site	# Alleles	Allelic Range	Ho	He	Allelic Richness	Gene Diversity	Theta (H)
12A1	2	1	0.28571	0.26374	1.264	0.262	2.07494
6A1	2	1	0.25000	0.25000	1.250	0.250	2.16667
4B1	2	1	0.20000	0.20000	1.200	0.200	2.62500
3A1	4	12	0.33333	0.39869	1.399	0.403	1.58562
C5-1	1	0	-	-	1.000	0.000	N.A
10-1	2	2	0.10000	0.10000	1.100	0.100	5.05556
2-1	3	8	0.50000	0.42632	1.426	0.422	1.54440
14A1	1	0	-	-	1.000	0.000	N.A
11B1	2	1	0.12500	0.12500	1.125	0.125	4.07143
R1-1	2	1	0.10526	0.10105	1.101	0.101	5.00412
S1-2	2	1	0.12500	0.11867	1.119	0.119	4.28066
Mean	2.091	2.545	0.18402	0.18031	1.180	0.180	3.15649
S.D.	0.793	3.652	0.15001	0.14318	0.143	0.143	1.35890

f)   Hr2.36

Site	# Alleles	Allelic Range	Ho	He	Allelic Richness	Gene Diversity	Theta (H)
12A1	6	9	0.66667	0.80303	1.803	0.817	2.66110
6A1	6	12	0.60000	0.88889	1.889	0.925	4.56250
4B1	5	16	1.00000	0.93333	1.933	0.917	7.53571
3A1	9	18	0.62500	0.90833	1.908	0.929	5.50500
C5-1	7	17	0.37500	0.82500*	1.825	0.857	2.96320
10-1	7	9	0.18182	0.77489*	1.775	0.805	2.36641
2-1	8	15	0.40000	0.87368*	1.874	0.900	4.03062
14A1	2	6	0.00000	0.66667	1.667	1.000	1.75000
11B1	4	8	0.33333	0.56061	1.561	0.583	1.52982
R1-1	3	3	0.25000	0.63741*	1.637	0.646	1.66340
S1-2	3	3	0.26471	0.60360*	1.604	0.609	1.58971
Mean	5.455	10.545	0.42695	0.77356	1.770	0.817	3.28704
S.D.	2.147	5.176	0.27698	0.12821	0.132	0.143	1.84544

The remaining measures of genetic diversity (heterozygosity (Ho), gene diversity and allelic richness) were subjected to permutation tests to specifically determine whether estimates of these parameters were higher in wild caught populations (group 1) than in hatchery stocks (group 2) in FSTAT. These tests were performed under several different conditions. Firstly, sample 14A was removed due to the small sample size ($n = 2$). Then differences were tested for significance over all loci, then removing locus Hr2.36 suspected of having the high frequency of null alleles, and then removing all loci with high null allele frequencies (RubCA, Hr2.9 and Hr2.36). These loci were removed as they may be under-estimates of genetic variation. Apart from the genotyping error

analysis (Table 7), deviations from Hardy-Weinberg equilibrium (see Table 8) suggest that heterozygosity is being grossly under-estimated for these three loci.

Table 9 shows the results of the permutation tests. The only measure of genetic diversity that showed a statistical difference between wild and hatchery stocks was gene diversity (Nei, 1987) for tests 1 and 2. It was expected by removing loci with high null allele counts, the difference may be more pronounced as it is likely that genetic diversity is being under-estimated at those loci. However, this was not the case, with the p value increasing as the number of loci removed from the analysis increased. This is likely to be a result of a reduction of statistical power due to the fewer number of loci involved in the permutation process.

So while real differences may exist, there is insufficient data to detect it when loci numbers are low. It should be noted that the test for differences of allelic richness was almost significant for the test with all six loci, suggesting that this measure may be useful when loci numbers are adequate. This would be a useful measure to keep for further analyses as it is specifically designed to account for unequal sample size, a situation that is likely to be common.

Table 9.   Results of permutation tests for three measure of genetic variation. Test 1 used all loci, Test 2 omitted Hr2.36 (the locus with the highest frequency of null alleles) and Test 3 omitted locus RubCA, Hr2.9 and Hr2.36 (all loci with high frequencies of nulls). Sample 14A was omitted from all analyses.

Test	Heterozygosity (Ho)			Gene Diversity (Hs)			Allelic Richness (AR)		
	Wild	Hatchery	p	Wild	Hatchery	p	Wild	Hatchery	p
1	0.459	0.403	0.141	0.655	0.512	0.007	1.644	1.511	0.069
2	0.448	0.398	0.164	0.539	0.456	0.017	1.536	1.456	0.093
3	0.367	0.389	0.655	0.404	0.367	0.134	1.408	1.367	0.272

4.2.2.6.   *Conclusions*

The data presented here show an obvious trend towards lower genetic variation in hatchery stocks compared with wild caught samples, even when sample sizes were relatively small from wild stocks. These results are not surprising given the relatively few broodstock used to produce the hatchery lines. However, only one or possibly two of the methods used to estimate diversity (gene diversity (Hs) and allelic richness) appear to be powerful enough to statistically detect differences. This effect is probably due to several factors: i) there was a high degree of variation in genetic diversity among wild populations; ii) a relatively high amount of diversity that managed to pass through the genetic bottleneck of five breeders used for this study; iii) level of statistical power may be insufficient in some cases.

Nevertheless, the loss of genetic diversity indicated here demands caution for further culture of the species in order to avoid potential problems of inbreeding. Also, it is suggested that when re-sampling takes place in the future, both gene diversity (Hs) and allelic richness are measured for all six loci.

(4)   Assessment of long term yields and cost-effectiveness of large-scale dispersed seeding of depleted wild fisheries stocks with large numbers of hatchery produced seed

This assessment is contingent on results of a re-survey of seeded and non seeded sites scheduled for late 2008 or early 2009 and subsequent analyses of these data to provide an estimate of yield per recruit i.e., survival of seeded juveniles through to a minimum legal//marketable size of 110 mm shell length. This estimate will in turn be fed into a bio economic model (see Section 4.4} and cost-effectiveness determined in conjunction with an evaluation of other indirect costs especially ecological and genetic impacts of fisheries enhancement using hatchery generated seed.

4.2.2.7.   References

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4.3.   Increasing indigenous capacity for abalone production

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4.3.1.   Aim

To provide the highest standard practical employment related training for Aboriginal people interested in working in aquaculture.

4.3.2.   Background and Need

Training for indigenous people formed one of four key objectives of FRDC 2001/033 "Development and delivery of technology for production, enhancement and aquaculture of black lip abalone in NSW". This objective was integrated into the Indigenous Fisheries Strategy (IFS), a NSW government initiative to facilitate the participation of indigenous communities in the NSW fishing and aquaculture industries.

The NSW fishing industry

The NSW fishing industry has a large aquaculture component. Commercial fisheries production in NSW in 2004/05 was valued at $131 million at point of first sale of which $48 million was for aquaculture (ABARE, 2006. Australian Fisheries Statistics 2005. ABARE, Canberra, ACT 2006). The aquaculture component of the NSW fishing industry is expected to grow in the future. In NSW production of the wild caught fishery has remained steady however if it mirrors most fisheries around the world is expected to decline in the future. However, aquaculture has experience the largest growth in any primary industry in the state increasing by 15.7% in 2004/05 year. In the last decade Australian aquaculture has increased in value from $256 million to $743 million. Many indigenous communities are in the unique position in NSW in that they have access to high quality sites that are amenable to use in aquaculture.

The Indigenous Fisheries Strategy

The NSW Fisheries, Indigenous Fisheries Strategy is based on "key platforms" which the NSW Aboriginal Land Council stated must be central to future NSW Fisheries planning. One of the platforms is: "Employment: Aboriginal people and communities accessing training opportunities in aquaculture industries."

As a result one of the key strategies of the IFS implementation plan is to "Actively promote Indigenous involvement in aquaculture through community based workshops, by offering professional advice and by helping to develop and deliver training programmes".

Specific initiatives of the IFS implementation plan include:

"Promote Indigenous involvement in shellfish aquaculture"

"Work with NSW TAFE and other training organisations to encourage employment related training for Aboriginal people interested in working in aquaculture"

Training at Tomaree Aquaculture Facility

One of the impasses to greater participation by indigenous communities in aquaculture is the lack of relevant aquaculture training. While there are TAFE courses available, aquaculture is a new and emerging industry and it is difficult for TAFEs to provide practical components in their courses that provide up to date training. The NSW fisheries Abalone research facility at Tomaree practices state-of-the-art land based flow-through aquaculture specialising in marine invertebrates. Early in 2004 the IFS working group vetted and accept a proposal for the Tomaree facility to be the focus of aquaculture training and promotion to achieve the goals of the IFS Implementation Plan.

4.3.3.   Methods

Site tours: promoting shellfish aquaculture and training

Tours of the Tomaree aquaculture facility were held in late 2003 and 2004. These tours were used to introduce indigenous community groups and TAFE students to shellfish aquaculture and to encourage participation in the practical course and/or the work experience programme at Tomaree.

Development of training programme

In late 2003 and early 2004, several meetings with representatives of Hunter TAFE took place to tailor the Tomaree practical training programme to the needs of NSW TAFE students. An outline of this training course was produced and vetted by the Tomaree TAFE and the IFS working group. Three topic areas were identified as important and incorporated into the training plan: Routine and maintenance skills, Aquaculture construction experience and Shellfish breeding and larval husbandry.

Recruitment of students

Two strategies were used to maximise participation in the aquaculture training.

1.   TAFE groups and Indigenous communities were directly invited to participate in the tours and then practical course.
2.   During tours of the Tomaree facility convenors and teaches of Indigenous aquaculture courses were individually encouraged to have their students participate in the Tomaree training course or work experience.

Work experience

For a less structured training experience Indigenous TAFE students and other Indigenous peoples are encouraged to participate in work experience at the Tomaree facility to apply the skills learnt during the training programme and or learn new skills.

4.3.4.   Performance Indicators

Tours of the Tomaree abalone hatchery were well patronised by Indigenous groups (Table1). During 2004 14 indigenous groups totalling more than 75 people visited the facility. These included 4 groups of TAFE trainees. During all visits the facilities were inspected a one of the staff gave an introduction to land based flow-through abalone aquaculture. This was followed by a more detailed explanation of the basics of abalone culture. Visits to the abalone facility were in most cases followed by a tour of the Port Stephens Fisheries Centre. Here introductions were given to oyster and finfish aquaculture. All TAFEs offering aquaculture courses with indigenous students in the state made visits to the Tomaree facility.

Table 1.   Visits to Tomaree Abalone Facility by indigenous groups during 2004.

Name	Approx. no. of people
Barry Lillee	1
Indigenous Fisheries Working Group	12
Indigenous Fisheries Officers	2
Larry Russell	1
Pilliga (2 days)	4
Sea Right Conference Group	10
South Coast Aquaculture Working Group	8
Tomaree TAFE	14
Wollongong Aboriginal Aquaculture Corporation (2 visits)	7
Wollongong TAFE (2 days)	14
Total	73

Practical courses

The practical courses were a particular success at the Tomaree abalone hatchery. The goal of the training was to provide a flexible course to suit the needs of different groups. An outline of the three areas of study follows:

Daily Routine and Maintenance: This introduced the student to the nuts and bolts of aquaculture. Students were taught the daily routine of the facility and then with minimal guidance were expected to do these tasks each day. These tasks develop skills like adjusting feeding rates of animals, weighing out feeds, keeping records and measuring environmental parameters. Students were also taught how to do some of the less frequent maintenance jobs such as changing over and priming pumps.

Producing Larvae: One of the most complex operations of any aquaculture facility is getting animals to reproduce and rearing the larvae and juvenile animals. This unit gave students experience in spawning abalone, rearing their larvae to competence and inducing larvae to metamorphose. It was intended that this unit taught the students skills that can be applied to other species.

Construction: The farmer makes much of the equipment that is use in aquaculture. To give students experience and confidence in construction students made a piece of equipment that was used in the facility or can at their home TAFE.

Work experience: Several students took up the opportunity to visit for varying periods of from a few days to a week to gain a less structured experience at the facility.

Hatchery Manuals

The NSW Aboriginal lands council has stated that there are four key platforms that must be central to future NSW fisheries planing. Two of these are:

• Social and Economic Development: Aboriginal people and communities having access to economic opportunities in established and emerging fishing industries

- Employment: Aboriginal people and communities accessing employment and training opportunities in resource management and in the fishing and aquaculture industries.

One of the key impasses to achieving these goals in relationship to the aquaculture industry is access to training and the specialised knowledge needed for modern aquaculture. To complement the training programmes run at Tomaree abalone facility two manuals have been produced to provide a long-term knowledge resource. The two manuals cover a well established industry in the abalone hatchery manual an emerging industry in the sea urchin hatchery manual.

Abalone Hatchery Manual

In recent years, abalone farming has become one of the fastest growing aquaculture industries in Australia. To date aquaculture production of abalone is being developed primarily in South Australia, Victoria, Tasmania and Western Australia. It is projected that national value of aquaculture abalone production will be 150 million in 2010. NSW is well situated to start its own abalone aquaculture industry.

This manual was commissioned by Indigenous Fisheries Initiative of the NSW Government as a practical guide to commercial scale seed production of blacklip abalone (*Haliotis rubra)* in NSW. Methods and equipment are based on those described by Hone *et al.*. 1998 with more recent refinements developed by Dr Arthur Ritar and Mark Grubert in Tasmania during the course of the Fisheries Research and Development Corporation (FRDC) Project 2000/204 and in NSW during the course of additional projects supported by the Fisheries Research and Development Corporation namely FRDC projects 1998/219 and 2001/033.

The manual provides specialised instruction on how to collect and reproductively condition blacklip abalone broodstock, how to induce them to spawn and how to hatchery rear their young though larval and early juvenile (spat) stages to an age and size suitable for on-farming or for seeding depleted fisheries. With minor adjustments to methods and equipment, the manual should also serve as a useful production guide for most temperate species of abalone. There is an emphasis on year-round production of spat as opposed to the seasonal production practiced to date by commercial abalone farms in Australia. In view of the scarcity and high cost of coastal sites with access to marine waters in NSW, the techniques described are geared to efficient and intensive production of abalone spat using a small area of land. These techniques in some respects are quite different to those generally practiced to date by commercial abalone farms in Australia.

This manual is not however intended to give complete coverage of all aspects of blacklip abalone biology nor hatchery production technology. There is a large amount of such information published both in Australian and overseas in relation to blacklip and other abalone. The Table of Contents for the manual appears below:

- *Contents*
- *Acknowledgments*
- *Introduction*
- *How to use this manual*
- *Recommended companion manuals and reports*
- *Chapter 1 – Abalone biology*
- *Chapter 2 – Broodstock collection and husbandry*
- *Chapter 3 – Planning and implementing breeding programs*
- *Chapter 4 – Induction of spawning and fertilisation of eggs*
- *Chapter 5 – Incubation of eggs and larval rearing*
- *Chapter 6 – Nursery production*
- *Chapter 7 – Management of nursery raceways and weaning*
- *Chapter 8 – Intensive on-rearing in shallow raceways*
- *References*

- *Appendices*
- *Glossary*

Sea urchin culture is an emergent world aquaculture industry. The roe of both male and female sea urchins are by weight one of the most valuable seafood products. The majority of Sea Urchin roe is consumed by the Japanese market and has been entirely reliant on wild caught animals. Since the 1980's wild stocks of sea urchins around the world have been in rapid decline. This has lead to interest around the world in the aquaculture of sea urchins. Australia and particularly NSW is in a good position to develop a sea urchin industry because of the presence in its waters of the sea urchin, *Tripneustes gratilla*. *T. gratilla* is one of the most sort after urchins by the Japanese market and is of particular interest to aquaculture because of its fast grow rate compared to many of the marketable urchins from around the world.

A small amount of research on producing *T. gratilla* spat has been conducted at the Tomaree facility and the knowledge garnered from this research has been put together in an easy to access form in the Sea Urchin Hatchery Manual. The manual begins with a brief description of some of the equipment needed to raise urchin larvae, offers tips on hatchery hygiene, describes how to collect and spawn sea urchins, and then explains in an easy to read manner how to raise the sea urchin larvae to competence. Finally, the manual offers suggestions on how to settle large numbers of competent larvae. Following the Sea Urchin Hatchery Manual are two scientific papers that have been written by the staff at the Tomaree facility and submitted for publication in international journals on the settlement of sea urchin larvae and on improving the palatability of artificial sea urchin diets. The Sea Urchin Hatchery Manual is attached as Appendix 9.5.

4.4.   Development of a bio-economic model as an aid to cost-effective fisheries enhancement of New South Wales blacklip abalone, *Haliotis rubra* (Leach) fishery

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Critical post release survival data needed for input into this model are subject to the completion of ongoing research (see Section 2.2.2) in 2 to 3 years time. A separate report for this objective will be provided as soon as practicable after that time. A major discussion paper prepared on this topic, incorporating preliminary results from the current project (FRDC 2001/033) and its precursor (FRDC 1998/219) was prepared for the World Congress of Malacology held in Perth, Western Australia in July 2004 and has been subsequently published in the Journal of Shellfish Research in 2006, Volume 25 (1).

4.4.1.   IN PURSUIT OF COST-EFFECTIVE FISHERIES ENHANCEMENT OF NEW SOUTH WALES BLACKLIP ABALONE, *HALIOTIS RUBRA* (LEACH) FISHERY

4.4.1.1.   Abstract

The diverse and often complex essential components of successful marine stock enhancement are briefly reviewed. Progress on a project to enhance the New South Wales blacklip abalone (*Haliotis rubra)* fishery is discussed to demonstrate that for sedentary reef invertebrates such as abalone, successful enhancement entails an understanding of recruitment and production limiting factors. These include age and size specific growth and mortality rates. These variables encompass the influence of other species within reef ecosystems, especially predators and species that compete directly with them for space, shelter and food. This discussion is also used to demonstrate that such difficult projects can be facilitated and refined using a simple bio-economic model centred on two interdependent tasks. The first is to minimise net costs per unit of additional sustainable production generated by enhancement. The second is to determine the minimum amount of additional sustainable production required to render a project cost-effective. This first task has, as expected, proven complex and its resolution protracted and expensive, requiring innovative biotechnology as well as a comprehensive knowledge of the natural biology of blacklip abalone. This discussion principally reflects the perspective of the direct potential; beneficiaries of abalone fisheries enhancement, namely commercial and recreational fishers and does not deal extensively with wider views and interests of other stakeholders, namely relevant government agencies and NGOs and the general public.

4.4.1.2.   Introduction and Background

Generalised theory and practice of marine fisheries enhancement

Blankenship and Leber (1997) identified ten key components as essential for responsible marine stock enhancement. These included the need to: 1) prioritize and select target species for enhancement; 2) develop a species management plan that identifies harvest opportunity, stock rebuilding goals, and genetic objectives; 3) define quantitative measures of success; 4) use genetic resource management to avoid deleterious genetic effects; 5) use disease and health management; 6) consider ecological, biological and life-history patterns when forming enhancement objectives and tactics; 7) identify released hatchery seed and assess stocking impacts; 8) use an empirical process for defining optimum release strategies; 9) identify economic and policy guidelines; and

10) use adaptive management. Blankenship and Leber (1997) reviewed three finfish case studies to verify that this responsible approach to marine stock management is practical.

To address these shortcomings, the authors proposed a flow-chart implementation model comprising four sequential steps: 1) review all relevant ecological and stock status management fisheries information; 2) make a comparative evaluation of all relevant fisheries management tools with a potential to meet targeted objectives; 3) instigate a scientifically based pilot enhancement program with clear appropriately targeted objectives; 4) if warranted by outcomes of the pilot program, initiate a follow-up full scale commercial program.

What follows is a discussion of a research project that has attempted to achieve sustainable and cost-effective enhancement the blacklip abalone (*H. rubra*) fishery in New South Wales (NSW), using hatchery-produced seed. This research, reported in detail by Heasman *et al.* (2004), has collectively addressed most of the important issues and elements identified by Blankenship and Leber (1997). Its implementation over a three year period generally conformed to the first 3 sequential steps prescribed by and Molony *et al.* (2003). Towards the end of the third year of pilot seeding operations (step 3), an extensive reappraisal of the project was made. This comprised a repeat of steps 1 and 2 (review of all other relevant fisheries biology and management information) and incorporated a wealth of practical experience gained, together with findings of an extensive array of complementary laboratory and field-based larval and juvenile seeding experiments. It also culminated in the development of bioeconomic benefit and cost and risk assessment model.

Previous attempts to enhance wild stocks of abalone with hatchery-produced seed

The efficacy of enhancing wild stocks of abalone with hatchery-produced seed has been investigated both internationally and within Australia. As stated by Shepherd *et al.* (2000), "Release of larvae and juveniles has often been proposed as a panacea for rehabilitating depleted or over exploited reef". Seeding of juveniles has been practised in Japan for several decades with some success (Kojima 1995; Masuda and Tsukamoto 1998). Larval release has been practiced sporadically in Mexico since the 1960's (Ortiz-Quintanilla 1980). Prior to this study, research in NSW into the efficacy of previously developed techniques for the release of seed abalone was limited to larval releases in Twofold Bay by a company Abalone Shellfish Enterprises Pty Ltd. (Keesing *et al.* 1994). Although survival of the larvae was not monitored, a localised high density aggregation of sub-legal size abalone was observed at one release site several years later (Ross Werner, pers. comm. 1998). This information, though anecdotal, encouraged collaborative involvement of TACC quota holders in this project as mediated through the NSW ABMAC (Abalone Management and Advisory Committee).

Larvae of both *H. rubra* and greenlip abalone (*H. laevigata*) were released at different densities in South Australia by Preece *et al.* (1997). Generally low and strongly density-dependent survival in the range 0.02 to 7.8% occurred 6 to 7 d after settlement. Such strongly density-dependent rates of mortality of larvae and early post-larvae were also reported by McShane (1991) in relation to naturally recruited stocks of *H. rubra* in north eastern Victoria. On the basis of these reports, Shepherd *et al.* (2000) recommended against larval seeding as did Schiel (1993) based on similar results with seeding of *H. iris* (Paua) larvae in New Zealand.

In a review of experimental releases of 7 month old (12 mm) hatchery-produced *H. rubra* in Tasmania, Shepherd *et al.* (2000) concluded that very high rates of mortality over the first few weeks were due mainly to handling stress. They also concluded that high mortality up to one year after release was due to persistent density-dependent mortality factors and predator naivety.

Status of the NSW abalone fishery and scope for enhancement

The NSW abalone fishery is based entirely on *H. rubra* and is largely confined to the southern half of the state. Annual catch (Figure 1) peaked at about 1,200 t in 1971/72, and remained above 600 t through to the early 1980's. Since 1973 a succession of fishery management initiatives (Figure 1) have been implemented. These have included a reduction in effort through license regulation, limiting catch by quota allocation and introduction of size limitations. Regional fishing closures were also imposed between Port Stephens and Jervis Bay (35º 03´S; 150º 44´E) following a major depletion of stocks by the disease *Perkinsus* between 2000 and 2002. A TACC of 370 t was first introduced in 1989. Subsequently it has been progressively reduced to 333 t in 1996, to 305 t in 2000 and down to its current level of 281 t in 2003 (NSW Fisheries 2004).

A common anecdotal report of *H. rubra* fishers over this 30 year period is that many formerly productive areas of reef, typified by foliose algae (seaweed) and associated complex communities of fish and invertebrates including abalone, have undergone a transition to "barrens". These bare rock areas typically carry high densities of the black (= purple) sea urchin, *Centrostephanus rodgersii*, (J. Smythe, pers. comm.). Such observations are compatible with the findings of Andrew and Underwood (1992) that densities of *H. rubra* and *C. rodgersii* are negatively associated and by the finding of Shepherd (1973) that continuous grazing pressure, exerted by dense aggregations of *C. rodgersii*, can degrade complex community reefs. Andrew and O'Neill (2000) and Worthington and Blount (2003) estimate that barrens habitat constitute an average of 40 to 50% of near-shore reef areas in NSW.

While the huge extent of barrens habitat in NSW appears to offer scope for rehabilitation of depleted *H. rubra* stocks, natural recolonisation is probably limited by the combination of competitive exclusion by *C. rodgersii* and the very restricted dispersal of *H. rubra* larvae from their parents (Prince *et al.* 1998). Hamer (1982) and Andrew *et al.* (1998) demonstrated that habitat improvement by way of culling urchins from urchin dominated reef in Southern NSW resulted in rapid recovery to abalone densities typical of high producing reef within 3 to 4 years. The recovery process also included a dramatic increase in the coverage and biomass of foliose algae and an associated increase in biodiversity of floral and faunal assemblages. Nevertheless, urchin culling is very labour-intensive and colonisation is limited to small localised areas. Accordingly, the task of culling *C. rodgersii* from up to 50% of coastal reefs commercially fished for abalone in NSW that collectively comprise about 5000 ha, is daunting.

By contrast, mass hatchery production and release of seed *H. rubra* provides a potentially powerful means of rapidly enhancing depleted abalone sub-populations over extensive areas. Seeding also addresses other factors limiting recruitment and consequentially sustainable yields. Such factors include the combined effects of commercial, recreational and illegal fishing pressure, and diseases such as *Perkinsus* that has devastated stocks north from of Jervis Bay to Port Stephens (Worthington 2002). Other recruitment limiting factors are pollution and competitive exclusion especially of settlement stage larvae and small post-larvae by a diverse array of large common surface grazers. These comprise other gastropods including several ubiquitous turban shell species and a comparable array of urchins additional to *C. rodgersii***.** In a recent experiment (Heasman unpublished data) high densities of the common tent shell (*Astralium tentorium*) or of *Turbo torquatus* in high densities were found to reduce yields of one week old *H rubra* post-larvae, seeded as larvae onto natural CCA (crustose coralline algae) rock settlement substrates, by 98 and 94% respectively. In the same experiment, the presence of either black urchins (*C. rodgersii*) or adult abalone reduced post-larval yields by similarly high margins of 90% and 78% respectively.

4.4.1.3. *Seed Production and Deployment Technology*

<u>*Improved year-round availability of ripe broodstock for induced spawning and hatchery production*</u>

Attempts to immediately produce viable eggs from wild-caught adults were unsuccessful, with a significant number of viable eggs (1.5 million) being produced on only one of 42 occasions. Over the same period seven successful inductions of spawning were achieved using broodstock acclimatised over longer periods in ambient flow-through seawater tanks. These yielded 26.8 million eggs (mean fecundity 1.12 million eggs/spawner) that in turn yielded 13.3 million competent larvae. However, these spawnings and subsequent hatchery operations occurred at irregular intervals and were largely limited to the spring to early summer natural breeding season of *H. rubra* in NSW.

For the first time in Australia, greatly improved access to ripe, ready-to-spawn broodstock was achieved with captive stock conditioned in a recirculating seawater system operated at 15 ± 2ºC. These stock were originally collected from seven localities between Port Stephens, (32º 42´´ S; 152º 10 E), 150 km north of Sydney, to Disaster Bay (37º 15´; 149º 58' E) near the Victorian border. Half (17of 34) spawning induction attempts, spread throughout the year, were successful and yielded 59.3 million eggs. Mean spawning response rate using these conditioned broodstock was 11% (85 out of 785 females). Mean fecundity ($\pm$ s.e.) was 1.25 ± 0.49 million eggs/spawner (range of 42,000 to 4.0 million). A total of 24.0 million competent larvae were produced and used for larval seeding experiments or for nursery production of juvenile seed and related experiments. Yields of 7 – 8 day old competent larvae from eggs averaged 40% (range 8 to 71%) which was consistent with rates routinely reported by commercial hatcheries in Australia. Subsequent yields of 6 to 9 month old juvenile seed from competent larvae averaged 5.7% and varied greatly from 8 – 75%.

<u>*Hatchery and nursery technology development*</u>

The low and inconsistent yields of juveniles raised the need for better knowledge of and control over nursery production. A mean shell length of about 1.5 mm was identified as the minimum size needed to ensure trouble free weaning of post-larvae onto finely ground particulate diets (Adam and Amos P/L, South Australia). Post-larvae grew at an exponential rate regardless of season or seed density (Figure 2). Plate residence time from settlement to 1500 μm ranged widely. Batches reared in summer/early autumn required only 30 to 35 d. At the other extreme, winter and early spring reared batches required 50 to 72 d. Growth rates thus appeared primarily dependent on seasonal temperature.

An important feature of growth on diatom plates is that it continues exponentially until either food runs out (plates grazed out), in which case it abruptly stops, or when post-larvae reach a mean shell length of 1 – 2 mm at which time they can be successfully transferred to raceways for weaning onto artificial diets. As illustrated in Figure 3, production batches that were inadvertently allowed to exhaust diatom films after having reached the minimum weaning size stopped growing and starved before being harvested from nursery plates. Substantial and sometimes catastrophic losses of post-larvae occurred as a consequence of such episodes. This was probably due to combined stresses of starvation, anaesthesia and handling when harvested and transferred to shallow exposed raceway habitats for weaning. The size and age at which growth stopped and starvation began appeared inversely related to initial density of competent larvae seeded onto the plates.

Results of experiments conducted to improve the reliability and cost efficiency of large-scale production of juvenile H. rubra seed (Heasman et al. 2004)

Results of one experiment to investigate effects of variable larval density of 500 to 4,000 larvae per plate (0.15 – 1.2 cm^{-2}) on settlement, metamorphosis and subsequent growth on conventional nursery plates showed that yields of post-larvae (Figure 4) peaked after 4 to 7 d. They also showed that yields increased from 26% to 71% with increasing seeding density. However, from day 7 to 28 after seeding, survival across all seeding densities progressively converged to a common rate of about 20% that persisted until the termination of the experiment at day 56. Growth (Figure 5) remained independent of initial seeding density up to 14 d, averaging about 14 μm per day. Subsequent growth became progressively more density dependent. By day 56, growth rates at residual densities of 10, 100 and 1,000 post-larvae per plate averaged about 40, 30 and 22 μm/day respectively. Minimum mean shell lengths of about 1,400 μm at day 56, attained at the highest initial density of 4,000 post-larvae per plate, were nevertheless found to be acceptable for the purpose of early weaning of post-larvae. These improved techniques provide scope to increase output per plate from one to at least four batches per year and to increase yield densities to a consistent range of up to 500 – 1,000/plate, thereby greatly raising annual yield/plate/year up from 30 – 80 up to a range of 2,000 to 4,000 (Heasman *et al.* 2004).

In another experiment, effects of temperature on settlement, metamorphosis and early post-settlement growth and survival were investigated. Settlement rates on natural CCA coated rock settlement substrates ranged from 20 to 40%. Peak settlement and yields of post-larvae occurred at about 17°C (Figure 6). During the first week after settlement growth rates of post-larvae (Figure 7) increased progressively from zero at 10°C to a peak of ~30 μm/day at 23°C, but then fell dramatically back to zero with a further rise in temperature to 27°C. Effects of temperature on growth of four juvenile age/size classes of *H. rubra* (Figure 8 a-d)) showed that further downward shift in optimum temperature from about 17 to 15ºC occurs with progressive age and size in juvenile *H. rubra* (Heasman *et al.* 2004). Results also demonstrated that moderate to high rates of settlement and subsequent growth and survival could be achieved year-round on the central coast of NSW at mean monthly sea temperatures in the range 16 to 22ºC but that successful year-round hatchery-ambient temperature nursery production would be limited at more southern sites subject to sea temperatures below about 14ºC.

Transportation and storage of juvenile H. rubra

The need to transport and deploy batches of up to several hundred thousand 6 – 9 month old (5 – 15 mm) juvenile *H. rubra* up to 500 km from the hatchery prompted the development of suitable storage and transportation methods (Heasman *et al.* 2004). Key issues addressed were whether stock should be stored and transported wet or damp, optimum temperatures for storage and transportation and the maximum duration over which they can be safely stored and/or transported without compromising the post-release vigour and survival. Juvenile *H. rubra* (mean SL, 14 mm) previously held at ambient temperatures of 18 to 22ºC survived at acceptable rates (i.e., above 80%) for up to 48 h when stored damp in pure oxygen between 13 and 16ºC. The best 48 h survival rate of 95% was achieved with damp storage at 14ºC. Generally poorer rates achieved with wet transportation were ascribed to declining physiochemical conditions, especially dissolved oxygen that rapidly fell below an acceptable threshold of 95% saturation.

Field deployment and post-release growth and survival

Improved methods of seeding hatchery-produced *H. rubra* larvae and juveniles onto natural reefs were developed, and the effectiveness of these methods to deliver competent larvae and juveniles to reefs tested (Heasman *et al.* 2004). Several alternative methods of monitoring the settlement and early survival of larvae, post-larvae and juveniles within their respective release areas were developed. Field experiments were used to demonstrate the ability of juveniles to rapidly disperse

from points of release and provided greater insight into factors that must be considered when attempting to estimate long-term survival of seeded stock.

In excess of 24 million larvae and nearly 900,000 juvenile *H. rubra*, most falling within the "button" mean size range of 6 – 18 mm SL, were seeded to 57 sites within six locations along the NSW coast. The survival and growth of larvae and juveniles were monitored for periods up to 30 months. Short-term (< 2 months) sampling was used to investigate movement of juveniles and to compare alternative sampling techniques. The best survival for seeded larvae a year or more after release was 125 out of 1.1 million (1 in 8,800) recorded 553 days after release. As in earlier abalone seeding studies (Preece *et al.*, 1997) seeding of ready-to-settle larvae was shown here to be fraught with practical difficulties and with very low rates of survival projected to yield less than 1 in 20,000 to a legal shell length of 115 mm.

Juveniles were mainly deployed as clusters of 700 to 2,500 in predator protective release devices initially comprising CCA coated rocks within wire mesh cylinders. These were superseded by rectangular boxes comprising sections of PVC down-pipe. Mean survival rates 1 – 2 years after release ranged from 0 – 4%. Average growth rates were similar among and between sites with juveniles expected to reach maturity (> 90 mm SL) after 2.5 to 3.5 years, and projected to reach legal SL of 115 mm after 4 y. These low survival rates prompted a compilation and analysis of published age and size-specific mortality data for *H. rubra* (Table 1; Figure 9) that were used to estimate yield per recruit and related parameters for a wide range of age/size classes of seed from competent larvae to 6 year old adults (Table 2). A review of published ecological information on *H. rubra* revealed that although 1 to 4 week old wild post-larvae occur at natural densities of up to several $1,000/m^2$, and 6 month old juveniles at up to $30/m^2$, survivors of these groups converged to a common narrow density range of $1 – 3/m^2$ as 15 – 30 mm, 1+ year olds (Prince *et al.* 1988; McShane 1991; Shepherd *et al.* 2000). It was then postulated that if natural survival rates of 6 – 9 month old "button size" (7 – 15 mm) juveniles, through to the minimum legal size, of 1 in 20 – 30 are to be achieved with hatchery-produced seed, then such seed should be sparsely deployed at densities matched to the food based carrying capacity of CCA rock habitats of this size/age class of juveniles, rather than in large dense clusters as previously practised.

Evaluation of post-larvae as an alternative to competent larvae for seeding

Major practical advantages of using week old post-larvae rather than settlement stage larvae for seeding operations were also identified in three laboratory experiments (Heasman *et al.* 2004). Results (Figure 10) showed that post-larvae can be readily seeded at very high densities onto small CCA coated pebbles (vector rocks) and retained for up to 8 d without significant restriction of growth or ability to rapidly disperse into simulated small boulder habitats after release. Carrying capacity of CCA rock habitat declines reciprocally with exponential increases in the biomass of individual *H. rubra*, falling to densities levels of 1,000 to $2,000/m^2$ for 2 month old (1.5 mm) post-larvae (Figure 11), and by extrapolation, to levels of around $\leq 30/m^2$ for 6 month old 7 mm juveniles. The latter was consistent with above cited peak densities reported for equivalent wild *H. rubra*. Surface grazing on CCA thallus and associated epibiota remains the primary food source of juveniles up to at least 10 mm SL and is only then progressively supplanted by drift seaweed over the following 1 to 2 y in individuals up to at least 35 mm SL (Shepherd and Daume 1996). An important aspect of these findings is that they were consistent with the above-cited convergence of 1+ year old wild juveniles to densities of $1 – 3/m^2$. These observed carrying capacity limitations of CCA rock habitats occurred in the absence of other important density and survival limiting factors, namely competition from other surface grazers such as urchins and other gastropods, and predation especially by finfish especially wrasses (Shepherd and Turner 1985; Shepherd 1998), wirra cod (*Acanthistius ocellatus*) and morwong (*Cheilodactylus fuscus*) (Heasman – unpublished observations) and large invertebrates including, starfish, rock lobsters, large crabs and octopus (Shepherd and Breen 1992; Shepherd 1998). Accordingly, the carrying capacity of CCA rock

habitats can be largely ascribed to nutritional factors in juveniles up to 10 mm SL and an important but diminishing factor in larger juveniles to 35 mm SL and around 18 months of age.

On the strength of these findings, a final reef seeding experiment was conducted at Disaster Bay near the NSW/Victorian border as part of the preceding project FRDC 1998/219 (Heasman *et al.* 2004). Two deployment treatments; the first consisting of 10 evenly spread clusters of 100 juveniles, averaging 15 mm SL, and the second, a central cluster of 1,000 juveniles of the same size, were each deployed over three replicate 115 m^2 sites. This represented a targeted seeding density of about 9 juveniles/m^2 typical for this size/age class of juveniles on healthy productive reef (Prince *et al.* 1988; McShane 1991; Shepherd *et al.* 2000). A year later, mean survival across sites seeded with 10 x 100 juveniles was within the expected range for equivalent wild stock, namely 12.5% (range 5 – 23%) while that of the alternative clustered treatment was only 3.8% (range 0 – 9%).

Development of a Bio-Economic Model for Enhancing the NSW Abalone Fishery

Many of the above findings were used to develop a comparative bio-economic model for *H. rubra* fisheries enhancement. The model is based on the most fundamental of economic equations:

$$\text{Profit} = \text{Revenue} - \text{Total costs}$$

where:
Revenue = long-term average beach price for abalone x additional sustainable catch generated by seeding.
Total costs = costs of all elements of production and deployment of seed but not including those of monitoring the wider environmental consequences of seeding, especially impacts on plant and animal communities and on the genetics of extant wild *H. rubra* populations.
Key inputs, assumptions and rationale thereof associated with this model as follows:

Additional sustainable annual catch targeted is 300 t (= 1 million, 120 mm abalone @ 300 g). This is valued at AU$15M based on an assumed average beach price of AU$45/kg (AU$15 ea.) (ABARE, 2003). The rationale for nominating an enhancement target of an additional 300 t/year, is that it would restore the total commercial catch to a range of 500 – 600 t/year which approximates the median annual catch of the fishery since its the inception in 1960 (Figure 1). It is likely that enhanced production of *H. rubra* can only occur at the expense of other reef surface and drift seaweed grazers that compete with *H. rubra* for food and space. However, when it is considered that 300 t of additional *H. rubra* equates to about 0.6% of the biomass of its most important competitor, the black sea urchin, *C. rodgersii,* estimated at 40,000 to 50,000 t by Worthington and Blount (2003), such impacts are likely to be sustainable.

The assumed mean age and weight of released abalone at harvest are 5 years and 300 g respectively. This estimate constitutes a median growth rate for *H. rubra* populations throughout NSW, based on regional specific rates originally derived from tag and recapture size data reported by Hamer (1982) and re-evaluated by Worthington and Andrew (1997).

The five size/age classes of seed and associated assumptions were as follows:

1) *Competent larvae* It was assumed that only 1 in 20 000 seeded larvae would survive to market size adults in compliance with yield per recruit predictions presented in Table 2. The latter, including a mortality rate of ~95% in the first week, are based on published instantaneous mortality data for wild *H. rubra* presented in Figure 9 and assume that seeding does not significantly reduce natural recruitment. Apart from enormous logistical problems of deploying the requisite 20 billion larvae over hundreds of hectares of juvenile habitat by

SCUBA under calm sea conditions, the production of the 2 billion larvae required would entail the reproductive conditioning and induced spawning of about 30 000 captive female broodstock. The latter is considered impractical if not cost prohibitive.

2)   *0.4 mm week old post-larvae* It was assumed that postlarvae would be pre-seeded onto vector rocks thereby circumventing otherwise prohibitively costly and difficult production and deployment problems plus very high post-release mortality losses sustained by larvae and post-larvae during the first week in the wild. An assumed yield per recruit rate of 1 harvestable *H. rubra* per 2 000 seeded post-larvae is based of data presented in Table 2 and incorporates an experimentally determined post-settlement mortality rate of 50% in the absence of predators. This particular mode of seeding would require year-round reproductive conditioning and induced spawning of 3000 captive female broodstock. The latter, although daunting, is considered practicable and affordable. The most critical limitation of this seeding strategy is uncertainty surrounding the fact that it has not been experimentally trialed in the field let alone on a commercially significant scale.

3)   *2 mm, 2 month old postlarvae* An estimated yield per recruit of 1 harvestable *H. rubra* per 157 ex-diatom nursery plate 2 mm postlarvae is based on reported age specific natural mortality data presented in Table 2. Production of requisite 157 million 2 mm post-larvae needed to raise sustainable fisheries yields by 300 tonnes would be cost prohibitive using current commercial hatchery technology. Although the scale of such seed production dwarfs that of existing commercial hatcheries in southern Australia, it is nevertheless considered to be both practicable and affordable provided much more space, labour and cost efficient hatchery and nursery technologies developed in this project are employed. Using such technology would entail the reproductive conditioning and induced spawning of 3200 captive female broodstock. Again, the most critical limitation of this seeding option is uncertainty surrounding the fact that it has not been experimentally trialed in the field let alone on a commercially significant scale.

4)   *8 mm/0.1g, 6 month old juveniles ("buttons").* An estimated yield per recruit of 1 harvestable *H. rubra* per 26 "buttons" is based of mortality data of wild *H. rubra* data presented in Table 2. Production of the 26 million buttons required would entail the reproductive conditioning and induced spawning of 2600 captive female broodstock. As with the previous two alternative seed classes, this relatively large broodstock requirement is considered both practicable and affordable as is seed production using new high efficiency hatchery and nursery technology developed during this project. However unlike week-old post-larvae and 2mm post-larvae, commercial-scale low density dispersed seeding methods for this class of seed have been fully developed and very extensively trialed in the field over the past 5 years.

5)   *21 month old, 40 mm/10 g juveniles* An estimated yield per recruit of 1 harvestable *H. rubra* per 15 seed comprising 40 mm juveniles, is based of mortality data of wild *H. rubra* data presented in Table 2. Production of the 15 million juveniles required would entail the reproductive conditioning and induced spawning of 2900 captive female broodstock. As with the previous three alternative seed classes, this broodstock requirement, though relatively large is considered both practicable and affordable. The extremely large seed production requirement of 15 million 40 mm juveniles equates to 150 tonnes liveweight which is likely to be cost prohibitive being more than twice the annual output of Australia's largest producing farm in 2004/5 (J. Hall, King Island Abalone P/L, pers. comm.). Further factors mitigating against these large seed are daunting logistical difficulties of handling, transporting and dispersion in the field.

Information provided in Table 3 summarises and compares key logistical, technical and cost issues in producing and deploying the five alternative size/age classes of seed. It also compares utility of

producing each size/age class using either convention single annual batch hatchery production with much higher efficiency technology developed during this project. Predictions regarding use of "button size" juveniles as seed are particularly encouraging in that the potential margin for profit forecast is so handsome that break-even enhancement would still occur even if survival of hatchery-produced seed fell as low as 10% that of wild counterparts. Alternatively, if average survival rates of say 25 to 50% of comparable wild stock are achievable profitable seeding of depleted populations of abalone could be attained at much smaller scales of operation than applied to this model.

The model also predicts that seeding of larvae is likely to be unprofitable and also casts doubt on the economic viability of large-scale seeding of natural reef using advanced (18 month old, 40 mm) juvenile _H rubra_. This is not to say that cost-effective seeding of more advanced juveniles cannot be achieved under reduced competition for food and shelter from competitors and/or reduced predation. For example, R. Day (pers. comm.) reported 50% survival of green lip abalone (_H. laevigata_) one year after being seeded as 28mm SL juveniles onto reefs constructed from natural reef rocks in shallow, high food flux (drift seaweed) sea-grass beds.

4.4.1.4.   Recent Developments (also see Section 4.2.2)

All field experimentation has been confined to the Port Stephens area due to stock translocation limitations imposed by an outbreak of the disease _Perkinsus_ that devastated the region's _H. rubra_ stocks by an estimated 90% during the summers of 2001 and 2002 (Daly 2004). Continuing research being undertaken by NSW DPI Fisheries includes development of a novel method of achieving low density dispersed seeding. This has involved the design and manufacture of small predator protective release capsules that accommodate only 10 – 20 "button size" seed. These miniaturized deployment devices can be broadcast over entire juvenile habitats of depleted reefs during daylight hours from surface craft, thereby negating costly deployment by divers. A key design feature of the capsules is that they pack together to form complete level platforms. This feature together with the use of intensive light encourages in excess of 95% of the "buttons" to self-load into the capsules (Heasman –unpublished data). Once loaded, the "buttons" can be maintained within the capsules in good health for several days of storage awaiting either more favourable sea and weather conditions or off-road transportation to sites up to several hundred kilometres from the hatchery. The latest advance in this technology has been the development of two alternative environmentally friendly forms of the capsule. One is manufactured from a specially developed biodegradable resin and the other of aluminium that will corrode away in seawater within a year.

In late July and early August 2005, 55 000 "button size" (average SL 11 mm) juveniles were stocked into aluminium dispersion release devices. Approximately 560 devices containing an average of 16 "buttons" were seeded across each of six 1000 m^2 sites marked by surface buoys. All six seeded and an additional six unseeded control sites located on the southern side of the Tomaree Peninsula at Port Stephens. All sites were considered typical juvenile habitat comprising a high component of crustose coralline algae coated boulders immediately adjacent to locations identified by local commercial fishermen as having consistently supported good catches prior to _Perkinsus_ epidemics that decimated local stocks in 2000 and 2002. Broadcast seeding of the capsules from a 10 m vessel required only a few minutes per site. Aims of this long term experiment are to evaluate both the commercial utility of enhancing depleted stocks with hatchery produced seed and to assess genetic impacts of such releases.

Possible deleterious genetic consequences of seeding large numbers of juveniles produced in the hatchery from a small number of parents has also been addressed in the current project through the development and assessment of triploid _H. rubra_. Chemical induction techniques yielding up to 100% stable triploids were developed. These triploids were subsequently shown to be reproductively sterile, and although no growth advantages could be demonstrated in triploids up to

37months old and 70mm SL, yield of saleable flesh was 20% greater than in full sib diploids. (Lui, 2005). Furthermore, one particular treatment used to induce triploidy was found to have inadvertently produced significant numbers of tetraploid individuals paving the way for future research to produce 100% functionally sterile triploid progeny by fertilizing eggs from diploid females with sperm of a tetraploid males (Liu *et al.* 2004).

4.4.1.5.   *Conclusions*

Considerable progress towards cost-effective enhancement of the NSW abalone fishery has been made through reducing costs of production and transportation of *H. rubra* seed and from the identification of "button size", 6 to 9 month old juveniles as the most appropriate class of seed for enhancing depleted populations. The detection of a major apparent flaw in the use of clustered deployment of these juveniles and development of low density dispersed seeding technology is also a significant step forward. This will hopefully enable seeding to be aligned with surface grazing based carrying capacity limitations of CCA rock habitats in relation to juveniles up to at least 12 months old and 25 mm SL.

Nevertheless, many other major questions and issues remain to be addressed. Foremost is the issue of matching seeding operations with inherent capacities of individual depleted reefs to sustainably yield higher quantities of marketable 4+ year old adults. Division of the NSW abalone fishery into six geographical management zones (Figure 12) reflects a very steep south to north gradient in the abundance of *H. rubra*. Almost 80% of TACC quotas are taken on average from zones 1 to 3 that collectively fall within 150 km of the Victorian border. At the opposite extreme, catches from zone 1, that stretches 600 km north from Jervis Bay to the Queensland border, have fallen from around 20% of total commercial landings in the mid 1980's to only about 5% over the recent years (Anon. 2002). The relative importance of such interactive factors as recruitment, availability of food, primarily drift seaweed (Shepherd and Hearn 1983), competition for food and shelter, and differential mortality factors, including natural predation and fishing pressure, plus disease (especially *Perkinsus*) on this changing pattern of abundance, is not well understood.

Higher sea temperatures in the north are undoubtedly a greater constraint to full development of gonads and hence to successful spawning and recruitment in some years. These processes require at least 1200 degree days at moderate temperatures in the range 8 to 18°C (Grubert and Ritar 2003). Likewise, losses of up to 90% of stocks in the Port Stephens area of zone 1 in 2000 and 2002, attributed to stress-induced susceptibility of *H. rubra* to the disease *Perkinsus*, could, together with impaired breeding, be linked to increased water temperatures. This has also been suggested as a possible cause of the decline of another abalone fishery by Shepherd *et al.* (1998).

Future development of tetraploid *H. rubra* offers the prospect of using functionally sterile 100% triploid seed to enhance depleted reefs. In turn this could enable "put and take" ranching in NSW capitalising on large-scale cost efficient centralised production of seed. Home ranges of *H. rubra* and natural recruitment in relation to parent stock have both been shown to be limited to scales of several hundred metres in *H. rubra* (Hamer, 1982). Likewise, productivity of particular areas of reef can vary greatly over distances of hundreds or even tens of metres. Effective management protocols therefore need to be tailored to accommodate these fine spatial scales.

In addition to a put- and- take ranching of sterile triploid seed there appears good scope for rebuilding depleted natural populations of *H. rubra* in NSW. However in this case, seed will need to be produced in hatcheries from genetically matched parent stock in sufficient numbers to safeguard the integrity of natural gene pools, including preservation of rare or unique alleles. Rebuilding standing stocks of *H. rubra* to high levels at which sustainable natural recruitment can be restored will probably require integration of seeding with new and innovative fisheries management initiatives. Such initiatives will need to address apparent imbalances between *H.*

rubra and several of its major competitors, especially the black urchin *C. rodgersii* and other large and common grazing gastropods, especially *Turbo torquatus (*Gmelin,1791), *Turbo militaris* (Reeve, 1848) and *Astralium tentorium* (Jonas, 1845). This in turn can only be achieved through co management of the competing species (Worthington and Blount 1997). In contrast to *H. rubra, C. rodgersii,* and the other common gastropods cited above have to date not been subjected to substantial levels of fishing pressure. It is also likely that efficient reseeding protocols will need to be aligned to the patchy distribution of legal size adults. In practice this will entail targeted reseeding of juvenile habitat that lies adjacent to high yielding patches of reef. Such "hot spots" are well known to experienced commercial divers and are commonly characterised by fast growing abalone with elongated shells (Worthington and Andrew 1997).

4.4.1.6. Acknowledgments

4.4.1.7. References

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Table 1.   Published age related mortality data for wild *Haliotis rubra.* (See Shepherd and Breen 1992, for the mathematical relationship between instantaneous and proportional mortality).

Mean Age (age span)	Survival for period or per year	M (annualised instantaneous natural rate of mortality)	Source
25days(0 – 49Days)	0.5%	311/y	Preece *et al.* 1997
80 days (1 week to 5 months)	3%	6.6 – 10.2 (8.4)/y	McShane 1991
4 months (1 week to 8 months)	5.5%	1.4/y	Prince *et al.* 1988
1.25 years (6 months-2 years)	20%	0.7/y	Prince *et al.* 1988
2 years (1.5 – 2.5 years)	44% per year	0.81/y	Day and Leorke 1986
2.25 years (2 to 2.5 years)	41% per year	0.9/y	Prince *et al.* 1988
2.5 years (2 to 3 years)	64% per year	0.45/y	Hamer 1982 (Botany Bay stock)
3 years (2 – 4 years)	66% per year	0.42/y	Shepherd and Breen 1992
3.3 years (3 to 3.5 years)	45% per year	0.8/y	Prince *et al.* 1988
3.5 years (2 – 5 years)	70% per year	0.36y	Shepherd *et al.* 1982
4 years (2 – 6 years)	81% per year	0.21/y	Shepherd *et al.* 1982
4.3 years (4 to 4.5 years)	74% per year	0.3/y	Prince *et al.* 1988
4.5 years (4 to 5 years)	76% per year	0.274/y	Hamer 1982 (Eden Stock)
5+ years	82% per year	0.20/y	Beinssen and Powell 1979
5.3 years (5 to 5.5 years)	90% per year	0.1/y	Prince *et al.* 1988
6.3 years (6 to 6.5 years)	78% per year	0.25/y	Prince *et al.* 1988

Table 2. Estimated age related survival, yield per recruit and associated parameters for wild *H. rubra.*

Period/life stage, age (post se)t and size (mm)	Assumed rate of instantaneous natural mortality (M)	Proportion surviving period	Cumulative proportion surviving from larvae	Number of larvae required to yield 1 abalone to this age	Proportion from this age living to legal size	Number of seed of this age needed to yield 1 legal size abalone	Number per million seed deployed at this age that will reach harvest size	Source of survival data
Spawning to competent larvae	?	?						
Competent larvae to 8 days post set (0.3 – 0.4 m)	0.44/day	0.03	0.0300	33	0.0000	20704	48	Preece *et al.* 1997
8 – 19 days post set (0.4 – 0.6 mm)	0.042/day	0.63	0.0189	53	0.0016	621	1610	Preece *et al.* 1997
0.5 – 1.5 months (0.6 – 2.0 mm)	0.9/month	0.4	0.00756	132	0.0026	391	2556	McShane 1991
1.5 – 2.5 months (2 – 4 mm)	0.7/month	0.5	0.00378	265	0.0064	157	6389	McShane 1991
2.5 – 3.5 months (4 – 6 mm)	0.6/month	0.55	0.00208	481	0.0128	78	12778	McShane 1991
3.5 – 4.5 months (6 – 8 mm)	0.5/month	0.61	0.00127	789	0.0232	43	23232	McShane 1991
5.5 – 6.5 months (8 – 10 mm)	0.4/month	0.67	0.000850	1177	0.0381	26	38086	McShane 1991
0.5 – 1.5 years (10 – 35 mm)	0.91/year	0.403	0.000342	2920	0.0568	18	56844	Day and Leorke 1986
1.5 – 2.5 years (35 – 60 mm)	0.81/year	0.445	0.000152	6563	0.1411	7.1	141053	Day and Leorke 1986
2.5 – 3.5 years (60 – 85 mm)	0.42/year	0.67	0.000102	9795	0.3170	3.2	316973	Shepherd 1992, based on Shepherd and Hearn (1983)
3.5 – 4.5 years (85 – 105 mm)	0.3/year	0.74	0.0000755	13236	0.4731	2.1	473095	Derived from data in Figure 7
4.5 – 5.5 years (105 – 115 mm)	0.25/year	0.78	0.0000589	16970	0.6393	1.6	639317	Derived from data in Figure 7
5.5 -6.5 years (115 – 120 mm)	0.20/year	0.82	0.0000483	20695	0.8196	1.2	819638	Derived from data in Figure 7

Table 3.   Bioeconomics model summarizing requirements and associated benefits and costs of producing and deploying five age/size classes of *H. rubra* aimed at increasing the sustainable commercial catch in NSW by 300 tonnes (1 million x 120 mm abalone).

Age and size of seed	Assumed number of seed needed to yield 1 harvestable abalone averaging 120 mm and 300 g (from Table 2)	Estimated number of hatchery broodstock of this age/size needed to yield 1 million legal abalone valued at ~AU$15 M (assumes same survival rates as equivalent wild stock)	Estimated number of brood-stock spawnings needed to yield 1million legal abalone valued at ~$15M	Estimated annual operating costs using conventional hatchery production and seeding techniques plus general comments	Estimated annual operating costs using production and seeding techniques developed in this study *NB Estimated capital cost of hatchery = $1million*
Competent larvae	20,000	20 Billion	33,000 Assumed fecundity of 1.5M eggs/spawner and 40% yield of competent larvae from eggs = 0.6M/spawner	Logistical problems of huge numbers of broodstock demands and deployment of larvae appear insurmountable	Logistical problems associated with huge numbers of broodstock and deployment of larvae appear insurmountable
1 wk old post-larvae (0.4 mm)	2,000	≤2 Billion	≤3,300 Assumed 50% mean yield of PL's from larvae = 0.3M/spawner	Conventional hatchery and nursery technology is inappropriate	Promising option but technology of handling and deployment lacking
2 months ex plate (2 mm/0.002 g)	157	157 Million	3,200 Assumed 20% yield to this age from 1 week post set = 0.06M/spawner	Prohibitively costly ($6.28M)based on current commercial prices of 2 cents per mm = 4 cents per spat	$0.5M (see Table 4) Promising option but technology of handling and deployment lacking
6 month old juveniles (8 mm/0.1 g)	26	26 Million	2,600 Assumed 10% yield to this age from 1 week post set = 0.03M/spawner	Prohibitively costly ($4.16M) based on 2 cents per mm = 16 cents per spat	$0.665M (see Table 4)
21 months old juveniles (40 mm/10 g)	15	15 Million	2,900 Assumed 9% yield to this age from 1 week post set = 0.027M/spawner	Prohibitive facility would need to be twice as big as Australia's largest farm i.e., biomass of seed = 150 tonnes /year	Prohibitive facility would need to be twice as big as Australia's largest existing farm i.e., biomass of seed = 150 tones /year

Table 4. Estimated annual operating budgets for hatchery production and seeding of 26 million 8 mm juveniles and 157 million 2 mm *H. rubra* post-larvae. **Note**: these budgets incorporate new high efficiency hatchery and nursery technologies developed in this study but do not include depreciation on plant and equipment.

| *Salaries*		2 mm post-larvae	8 mm juveniles
(including 30% on-costs)		$	$
Seed Production			
Full time Manager		100,000	100,000
Full time senior fisheries technician		80,000	80,000
Full time fisheries technician(s)		60,000	120,000
Seed deployment and monitoring			
Casual assistants		30,000	60,000
	Sub Total	270,000	360,000
Travel and Accommodation			
20 trips for 3days by 3 staff @$120/d/person		21,600	21,600
Operating costs			
Power		60,000	80,000
Repairs and Maintenance		30,000	40,000
Vehicle operating		12,000	12,000
Post freight and packaging		5,000	5,000
Air fills		3,000	3,000
Telephone/email/www		3,000	3,000
Boat operating		10,000	10,000
Consumables		10,000	10,000
Chemicals/pharmaceuticals		3,000	3,000
Insurances		1,000	1,000
Food		1,000	10,000
Permits		1,000	1,000
	Sub Total	139,600	178,600
Interest on $1 m or $1.5 m for capital works			
loans at 7% pa		70,000	105,000
SUB-TOTAL		**Aus $500,600**	**Aus $656,500**
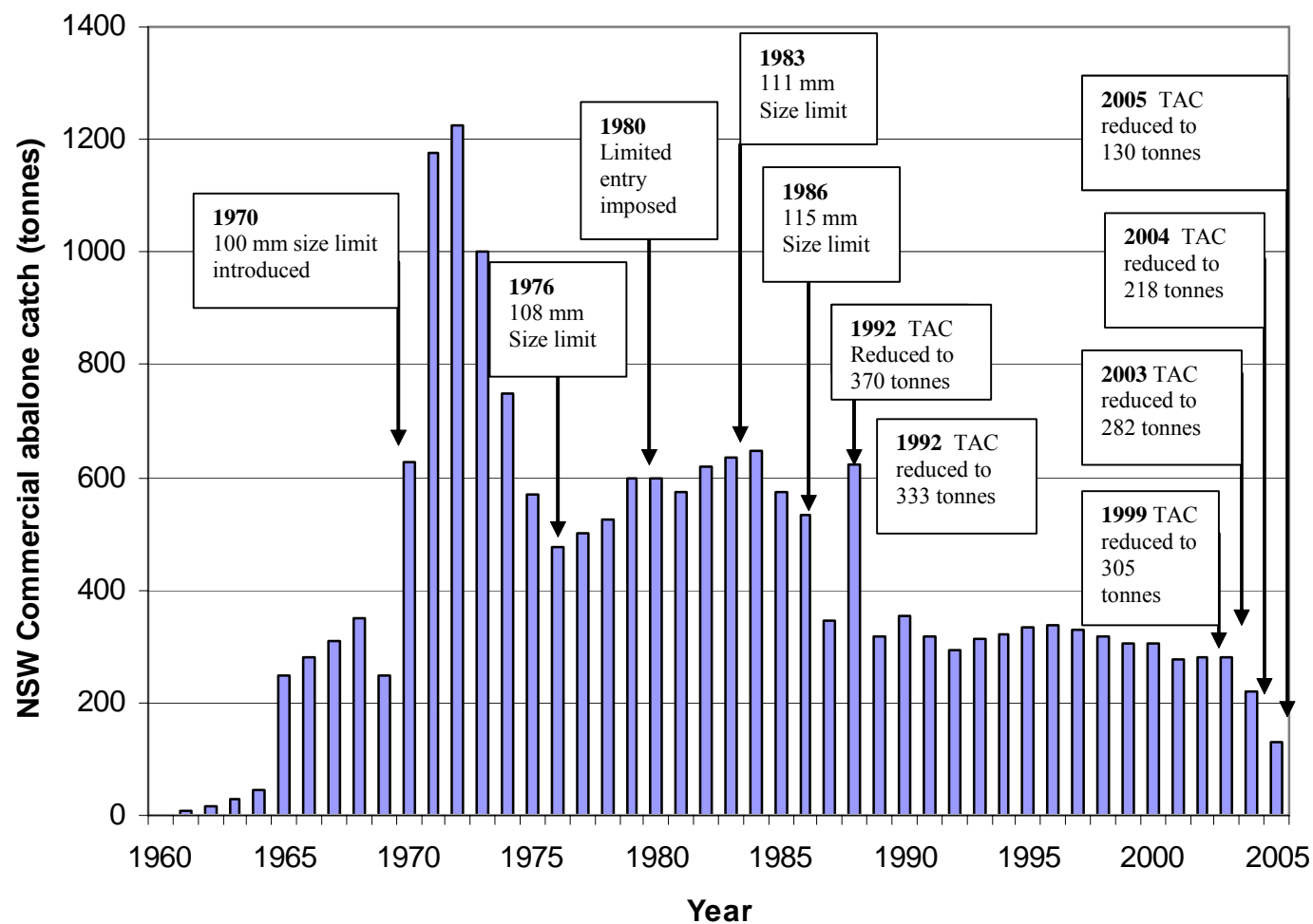
Figure 1. Annual commercial catch data and management initiatives for the NSW *H. rubra* fishery since its inception in 1960. (Source data: Anon 2002; NSW Fisheries 2004, G. Liggins, pers. comm.).
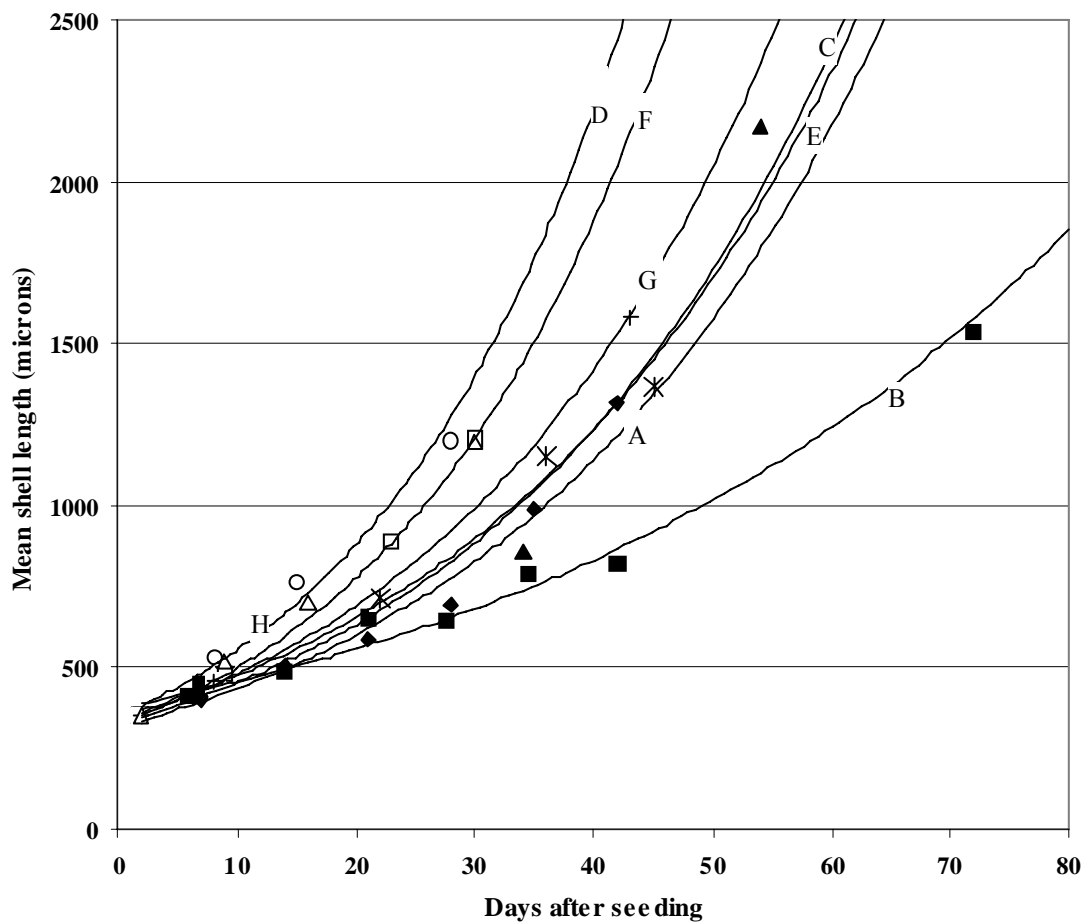
LEGEND:

Batch	Larval seeding density	Spawning Date	Growth season	Period to min. mean weaning size of 1500 microns	Mean growth rate microns /day	Best fit regression	R^2
A	5882/plate	23/07/98	late Winter early Spring	50 days	23 um/day	y = 314.71e0.0322x	0.9813
B	5147/plate	27/08/98	early to mid Spring	72 days	17 um/day	y = 373.24e0.02x	0.9751
C	1838/plate	25/12/98	Summer	45 days	26 um/day	y = 323.15e0.0335x	0.9808
D	7625/plate	20/01/99	late Summer	32 days	36 um/day	y = 348.05e0.0464x	0.9731
E	1472/plate	6/09/99	early to mid Spring	52 days	22 um/day	y = 345.04e0.0319x	0.9921
F	735/plate	14/09/99	mid to late Spring	47days	24 um/day	y = 320.56e0.0442x	0.9999
G	1323/pllate	24/11/99	early to mid Summer	41 days	28 um/day	y = 333.86e0.0362x	0.9988
H	2507/plate	1/02/00	late Summer	35 days	33 um/day	y = 337.31e0.0431x	0.9923

Figure 2.   Variation in growth of eight commercial scale batches of *H. rubra* post-larvae grown on conventional diatom plates. Note: growth was exponential in all cases (Heasman *et al.*, 2004).
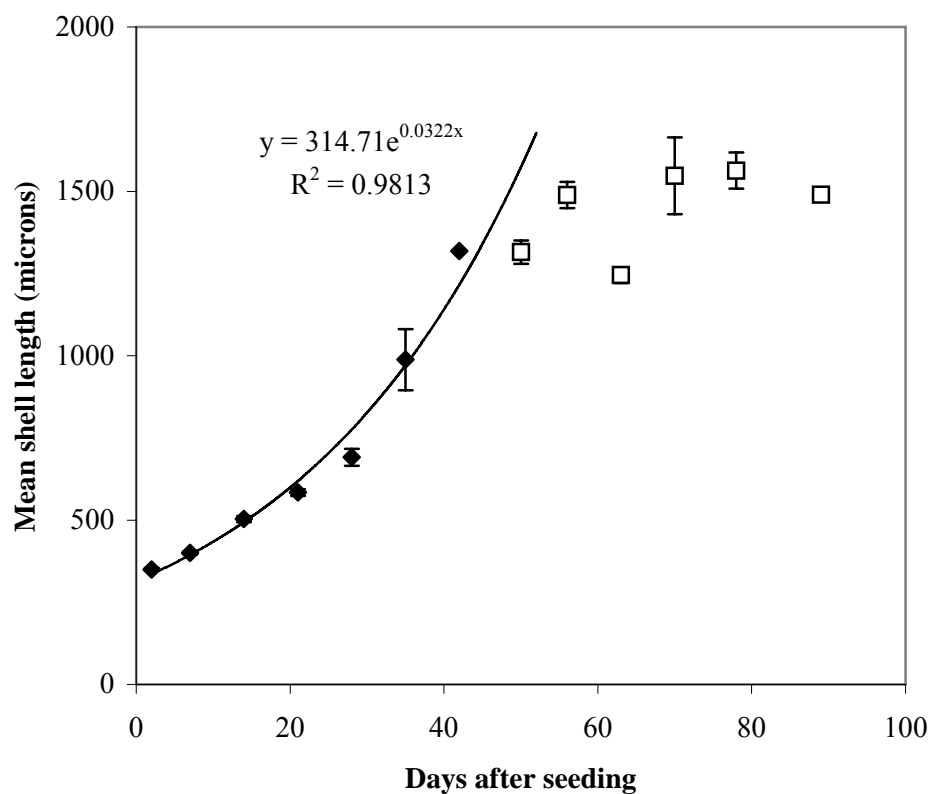
Figure 3.   Effect of over-grazing on growth of *H. rubra* post-larvae seeded onto conventional diatom plates (Heasman *et al.* 2004). Note: initial exponential growth (solid circles) succeeded by stalled growth and starvation (open circles). Bars are ± SE of means, *n* = 30.
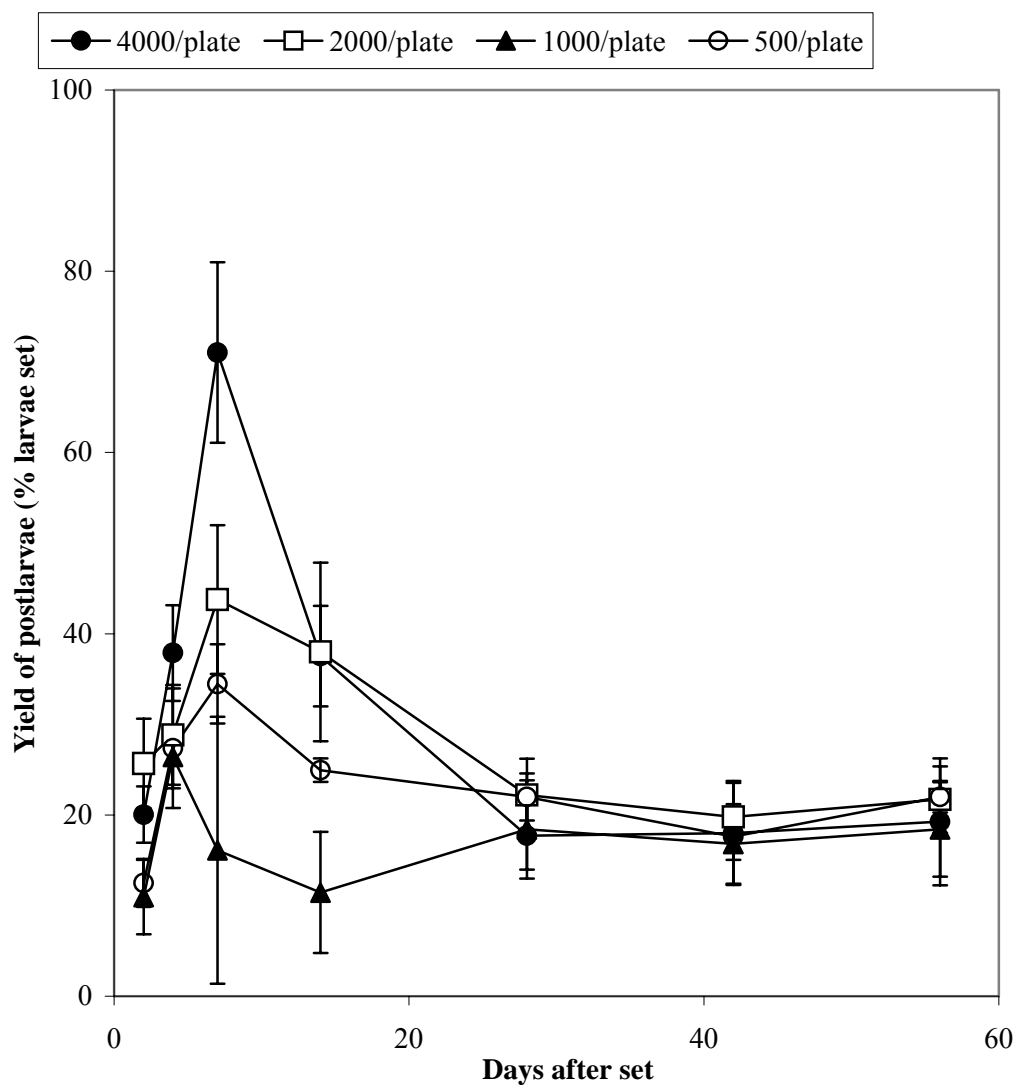
Figure 4. Effect of larval seeding density on the relative yield of *H. rubra* post-larvae seeded onto diatom plates. Bars are ± SE of means, *n* = 4 (Heasman *et al*., 2004).
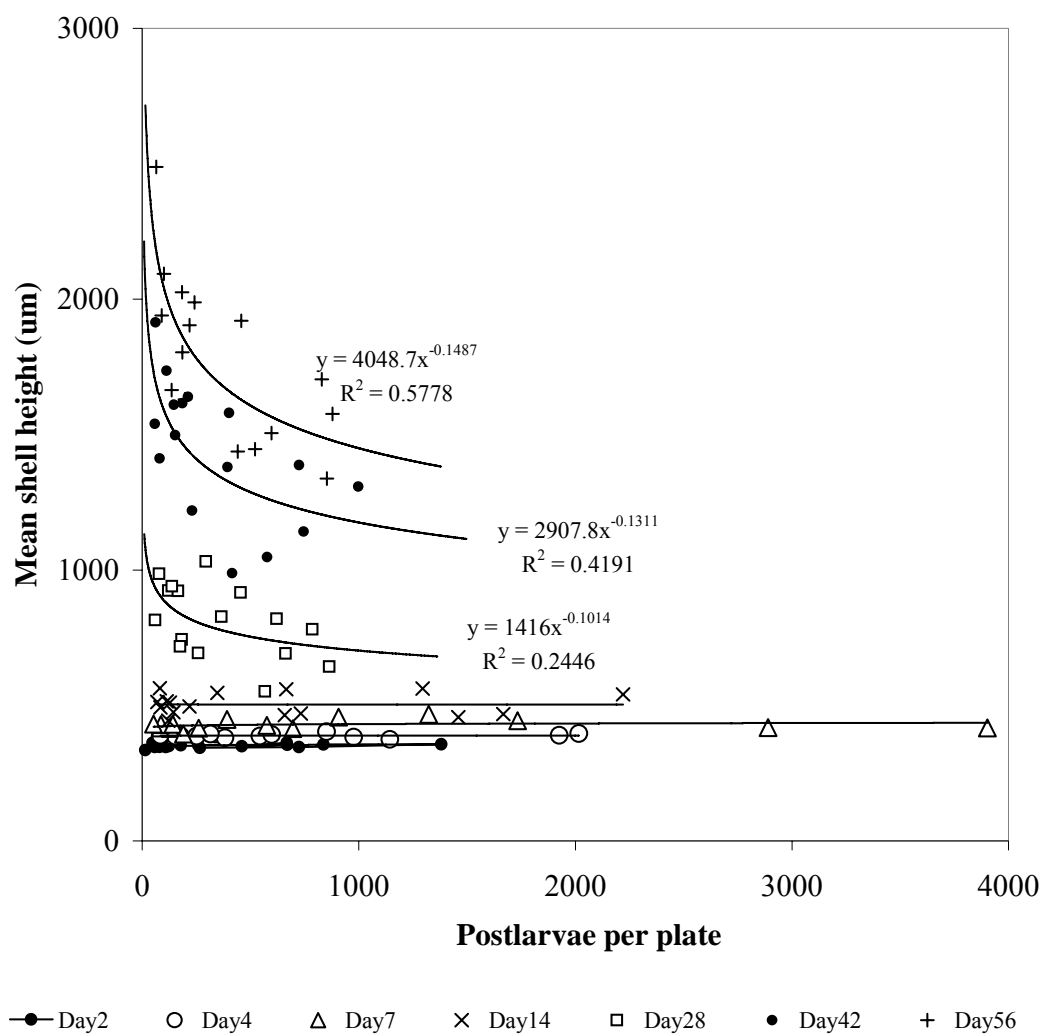
Figure 5. Effect of density on growth of *H. rubra* post-larvae on diatom plates 2 – 56 days after seeding as larvae (Heasman *et al.,* 2004).
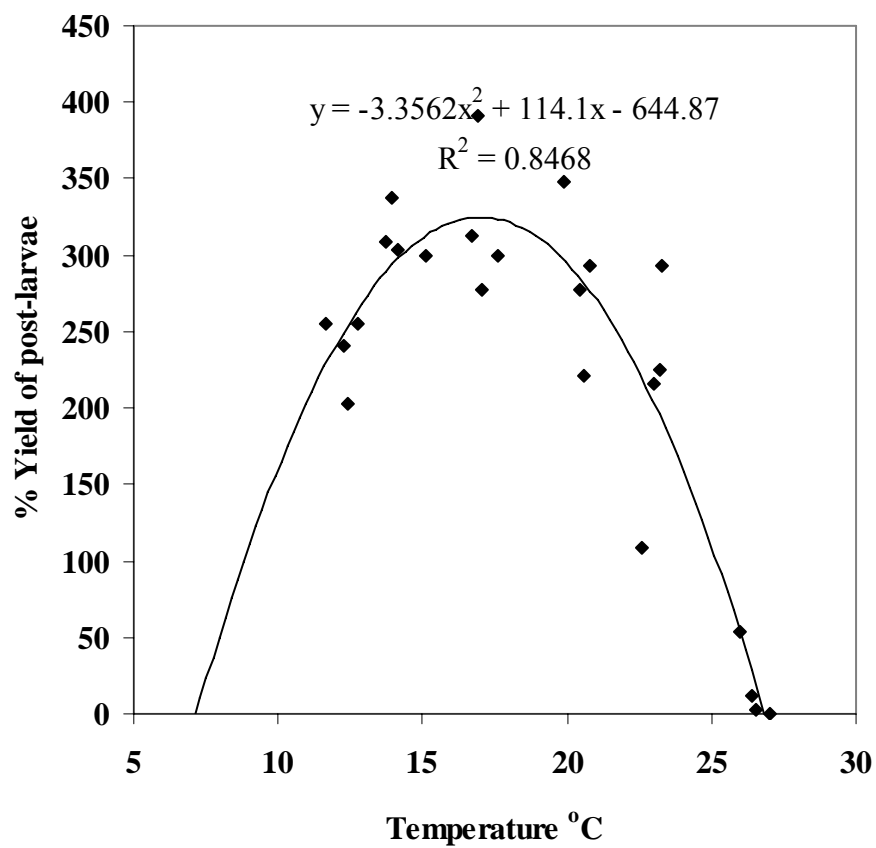
Figure 6. Effect of temperature on yield of day 6 *H. rubra* post-larvae as a percentage of larvae seeded onto crustose coralline algae coated rocks (Heasman *et al.,* 2004).

$$y = -0.0259x^4 + 1.7603x^3 - 44.63x^2 + 513.57x - 1892.2$$

$$R^2 = 0.9484$$
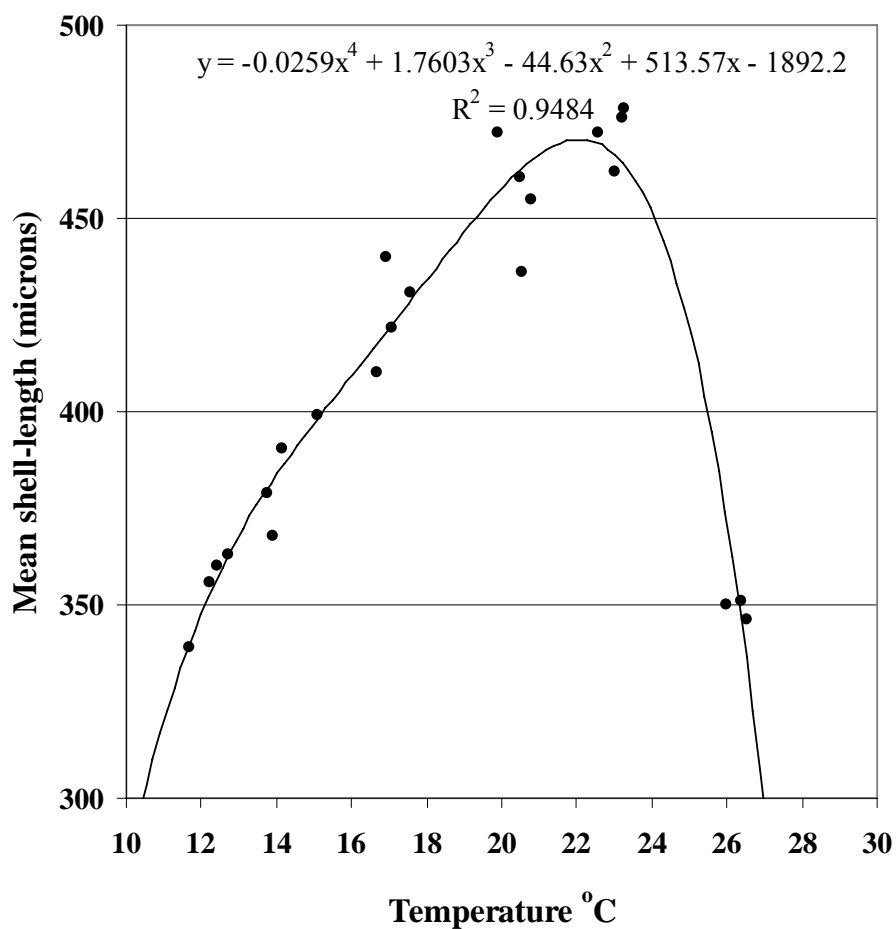
Figure 7.   Effect of temperature on growth of day 6 *H. rubra* post-larvae seeded onto crustose coralline coated rocks (Heasman *et al*., 2004).
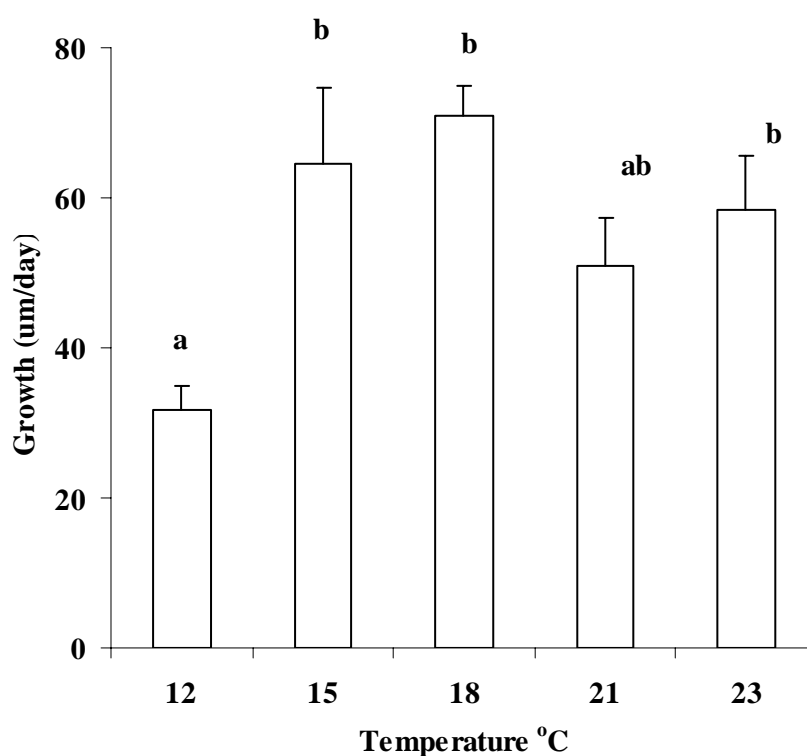
Figure 8a. Effect of temperature on growth in shell length of 3-month old, (2 – 6 mm juvenile *H. rubra.* Bars are +/-SE, *n* = 4 (Heasman *et al.,* 2004).
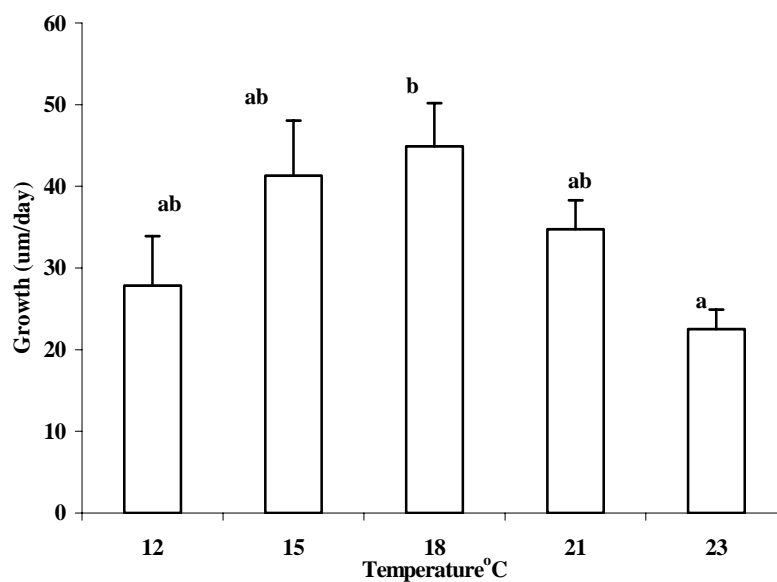
Figure 8b. Effect of temperature on growth in shell length of 7 month old, 6 – 8 mm juvenile *H. rubra.* Bars are +/- SE, *n* = 4 (Heasman *et al*., 2004).
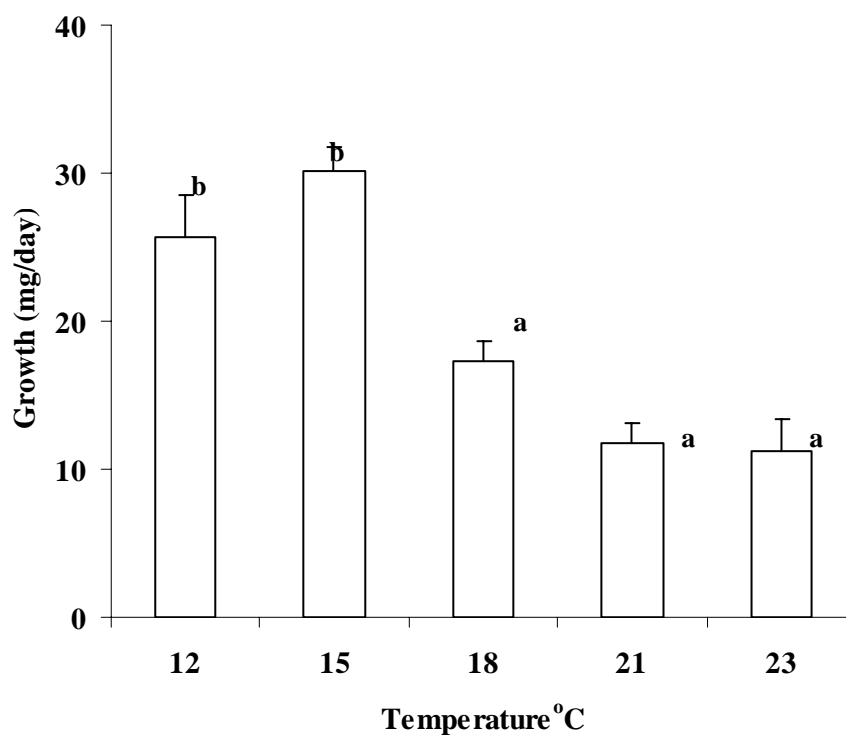
Figure 8c. Effect of temperature on growth in biomass of 17 month old, 20 – 25 mm juvenile *H. rubra*. Bars are +/- SE, *n* = 4 (Heasman *et al*., 2004).
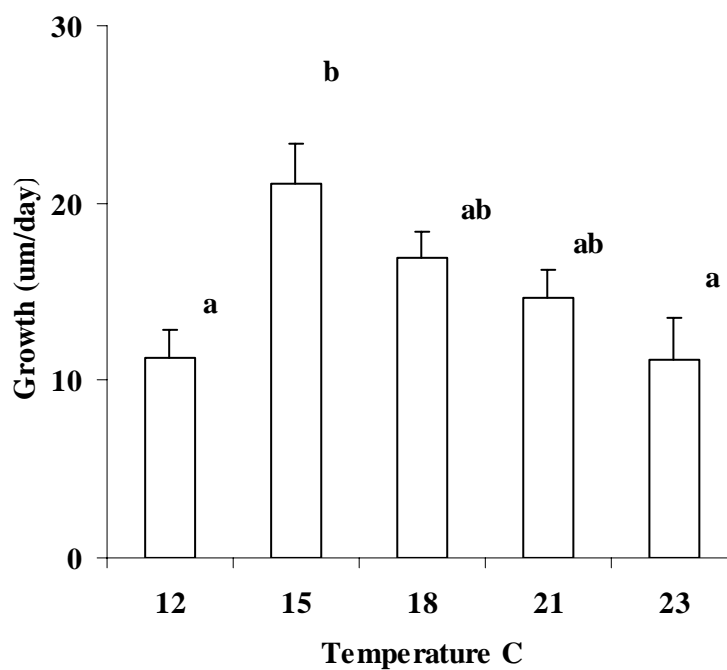
Figure 8d. Effect of temperature on growth in shell length of 30 month old, 50 – 60 mm juvenile *H. rubra*. Bars are +/-SE, *n* = 4 (Heasman *et al*., 2004).

The plot shows data points with axes: y-axis "Instaneous rate of mortality per year" ranging from 0.01 to 1000 (logarithmic), x-axis "Mean Age (years)" from 0 to 8. Annotation on plot: $y = 1.439x - 1.1772$, $R^2 = 0.9567$.
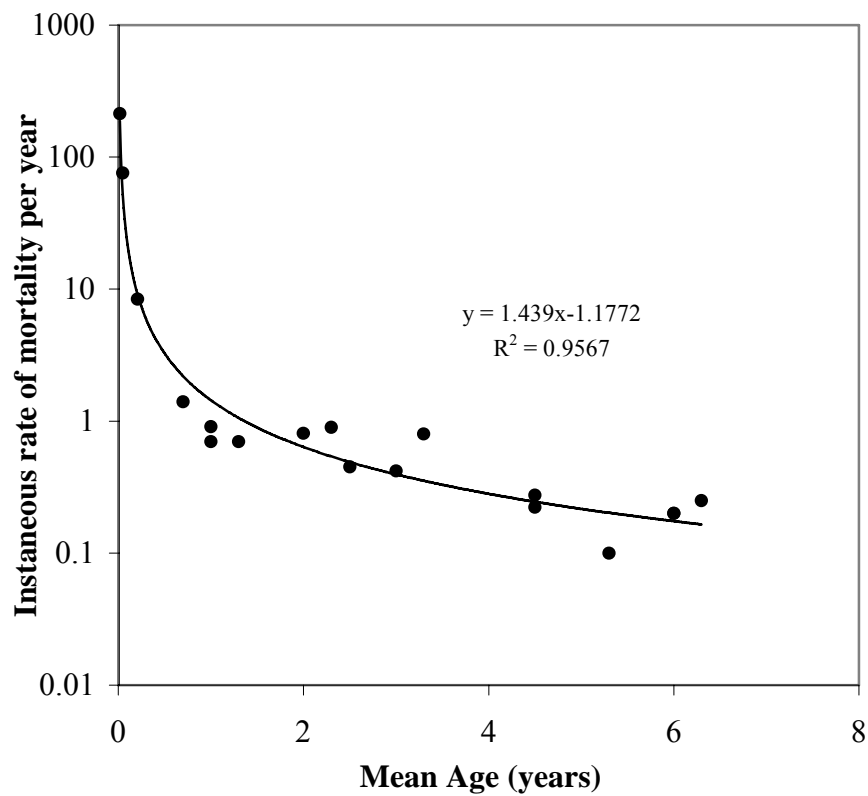
Figure 9. Age related annualised instantaneous mortality rates for wild *H. rubra* (see Table 1 for source of data).
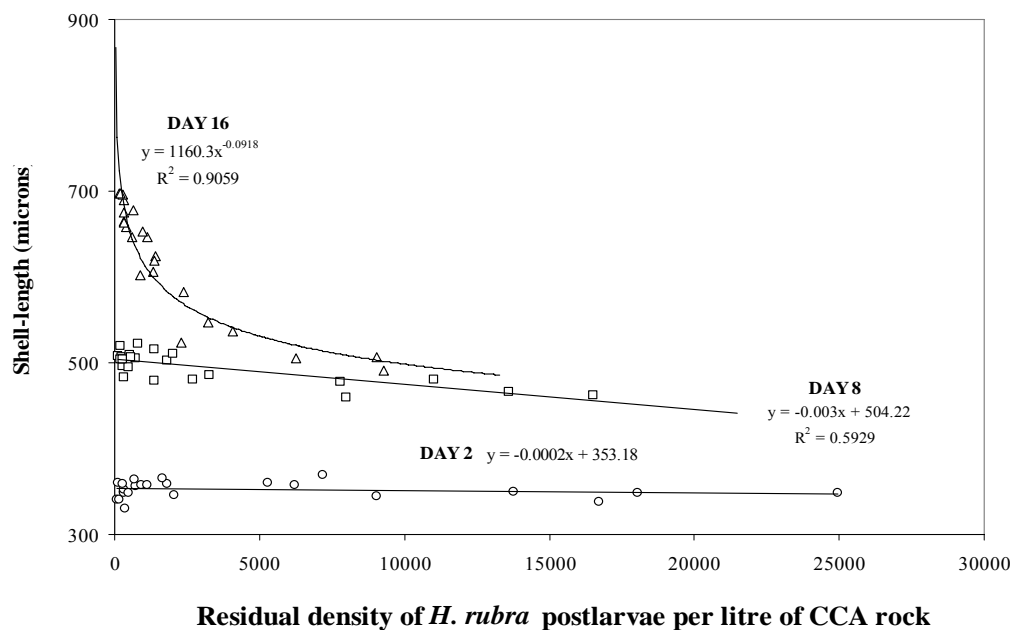
Figure 10.   Relationship between residual density and growth of *H. rubra* post-larvae seeded onto natural CCA settlement substrates after 2, 8 and 16 days (Heasman *et al.,* 2004).
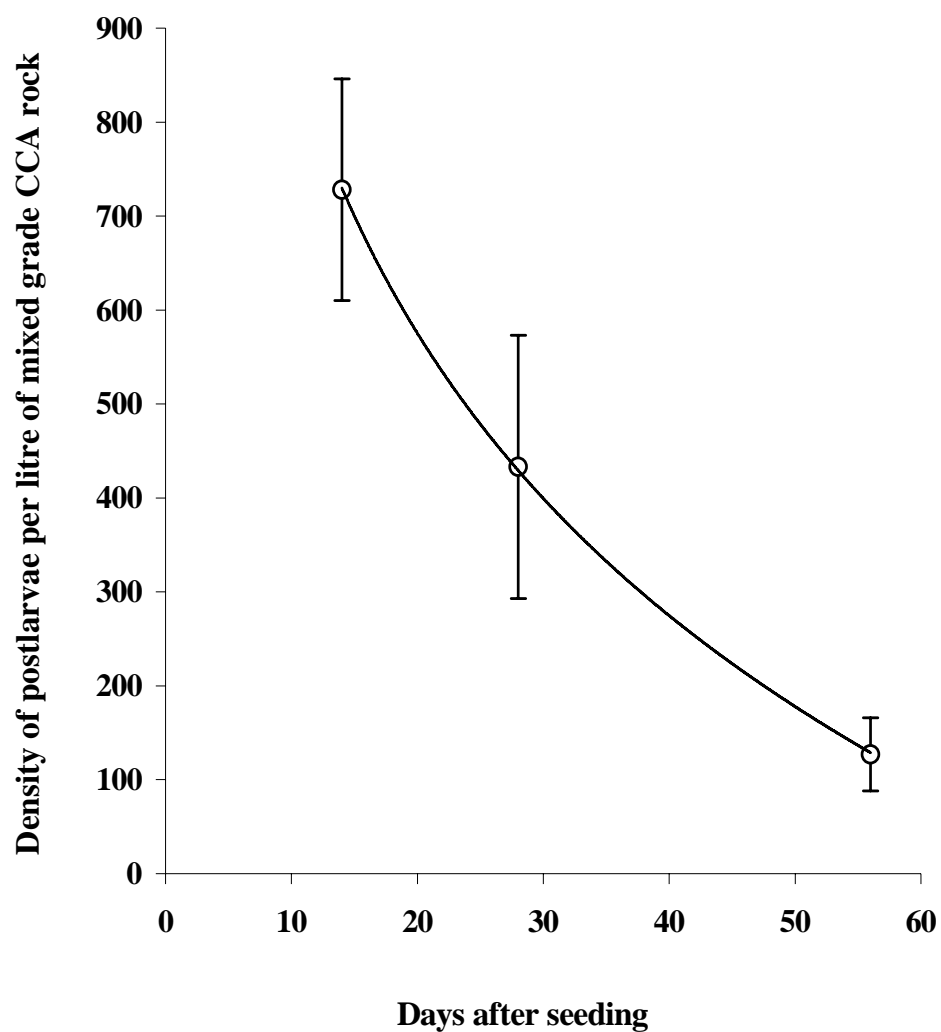
Figure 11. Residual density of *H. rubra* post-larvae per litre of CCA rock after 56 days. Bars are ±SE, *n* = 4 (Heasman *et al.*, 2004). **Note:** 100 post-larvae per litre of CCA rock represents 1,000 per m^2 of surface area.

Total catch in 2000-01 was 303,976 kg and in 2001-02 was 280,199 kg. (Catches for 2001-02 are in bold).

Catches in zones A-E were 1492 kg in 2000-01 and 3618 kg in 2001-02.

Note: Shaded areas are closed to abalone fishing

0   50   100
Kilometers

F 852 54

G 127 0

H 74 0

J 1161 199

K 698 581

Region 1 18837 **13515**

L 14434 **9064**

M 2161 **1334**

N 4570 **2847**

P 11031 **8583**

Q 5788 **4975**

R 2681 **1826**   Region 2 26230 **19564**

S 5913 **5695**

T 19218 **14309**   Region 3 62071 **50764**

U 36939 **30759**

V 17661 **16697**

W 23322 **21293**   Region 4 57256 **49746**

X 16273 **11756**

Y 57878 **55562**   Region 5 57878 **55562**

Y3 23815 **30400**

Z 57890 **60650**   Region 6 81705 **91049**

Figure 12. Abalone fishery zones and catches (kg) for financial years 2000-01 and 2001-02 (Anon. 2002).

5. BENEFITS

5.1. Objective 1: Produce and assess triploid *H. rubra* for farming and fisheries enhancement

An important practical outcome of this research was that fine tuning of chemical induction of tripoloidy with respect to 3 components of dosage level, time of initiation (time after fertilisation that eggs are first exposed to the chemical) and duration that the eggs are exposed to the induction chemicals, can yield commercially acceptable yields of healthy viable abalone with triploidy rates closely approaching 100%. Moreover, as these triploids were also shown to be reproductively sterile, their future use in put- and-take enhancement of depleted fisheries has the advantage of posing little if any risk to the genetic integrity of the depleted wild stocks.

Improved flow cytometric techniques that were developed for the verification of triploidy at various life phases of *H. rubra* have the distinct advantage over previous techniques of being able to be used to determine the ploidy status of individual postlarvae. A further advantage is that ploidy status can be determined non-destructively for juvenile abalone as small as 5mm.

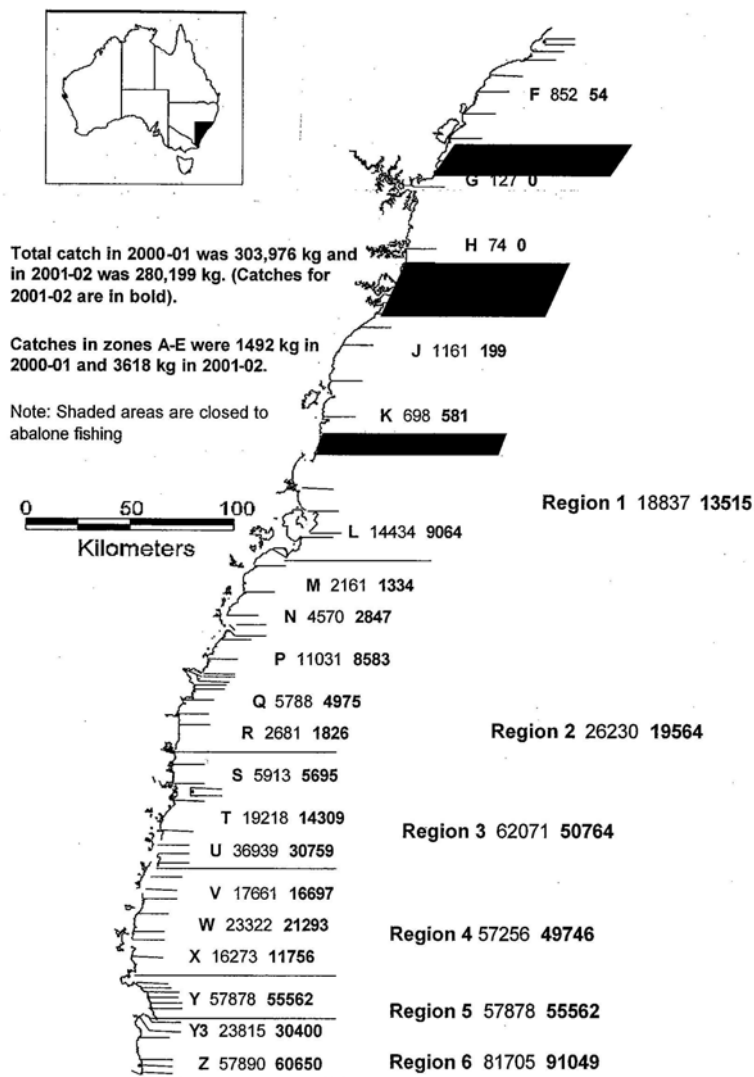Results of this research also showed that different triploidy inducing chemicals, in this case cytochalasin B and 6-DMAP, yield *H. rubra* triploids with distinctly different physiology and morphology. Up to three years of age no significant differences could be demonstrated in shell length, body weight, condition index and survivorship of sibling triploid and diploid *H. rubra.* However over the ensuing 6 months major differences in overall shell size and total liveweight emerged in one particular group of triploids but not in other triploid groups nor in diploid controls. Elaboration of these very encouraging results are constrained by non-disclosure provisions of a provisional patent registered by the FRDC and NSW DPI on behalf of stakeholders in April 2007. It can however be revealed that yields of saleable foot flesh of the particular triploid group referred to above was about twice that of all other groups at 3.6 years of age.

The greatest potential benefits of this research will be those that will hopefully lead to widespread adoption of higher yielding faster growing triploid abalone by up to 30 abalone farms currently operating in the four southern states of Australia.

5.2. Objective 2: Evaluate alternative methods of reducing high post release mortality rates commonly sustained by hatchery produced abalone

Complimentary experimental programs were used to investigate seeding density and scale effects on post-release survival of hatchery reared juveniles. The first program assessed the impacts of several factors on post-release the survival of cultured juvenile abalone. Button-size *H. rubra* were seeded at moderate densities of $100 – 120/m^2$ in a series of small-scale experiments. Poor apparent post-release survival suggests that the densities used (that are one tenth those used in earlier unsuccessful experimental seedings), were still too high, being about 10 times greater than those reported for equivalent naturally recruited wild stock. Concerns of possible adverse effects of artificial hatchery diets on the shell colour and vigour were however allayed after artificial diet reared stock were found to exhibit higher apparent post-release survival than normal coloured siblings reared on natural seaweed diets. Possible benefits of seeding hatchery reared juvenile under the predator protective canopy of urchin spines were dispelled by results of experiments to test this theory.

The second program culminated in a single large-scale release experiment initiated in July 2005. Juveniles housed in miniature predator protective release devices (capsules) were broadcast seeded

onto pre-surveyed juvenile habitats during daylight hours from a surface craft at densities thereby negating costly deployment by SCUBA divers. The release capsules that were specially developed for these operations accommodated only 10 – 20 "button size" seed each. A key design feature of the capsules is that they pack together to form complete level platforms through which seawater can be continuously passed. This feature together with the use of intensive light enabled more than 95% of the "buttons" to self-load into the capsules. Once loaded, the "buttons" can be maintained within the capsules in good health for several days of storage awaiting either more favourable sea and weather conditions or road transportation to sites up to several hundred kilometres from the hatchery.

This new deployment technique enabled seeding at densities of 8.6 – 10/m^2 that approximate those previously reported as most common for this size/age class in the wild. This experiment is being conducted on a much more expansive spatial scale (1000 m^2 replicate sites as opposed to 1 m^2 used in the first approach) and involves much larger numbers of seed abalone (around 8,600 to 10,000 per replicate site). This long term experiment being conducted on the Tomaree Peninsula, Port Stephens, NSW, is also attempting to evaluate environmental and genetic implications of seeding large numbers of cultured seed produced from a small number of parent stock on depleted wild populations. Definitive results of this experiment over the next two successive 3 to 4 year generations will be assessed and reported in due course. If successful, flow-on the benefits towards successful large-scale enhancement of the depleted abalone fisheries in NSW and elsewhere in southern Australia could be very substantial.

5.3.   Objective 3: Develop indigenous community capacity to undertake the production of seed abalone and use of that seed for cost effective and sustainable fisheries enhancement, ranching and farming of back-lip abalone

As part of the NSW Government Indigenous Fisheries Strategy, several initiatives were undertaken to develop an indigenous hatchery on the NSW Coast. This included a generous subsidy offered through an "Expression-of-Interest" to indigenous groups with a proposal, business plan and sufficient capacity to establish and effective hatchery. Unfortunately, no suitable proposals were received and the subsidy was not made available.

Tours of the Tomaree aquaculture facility held in late 2003 and 2004 were used to introduce indigenous community groups and TAFE students to shellfish aquaculture and to encourage participation in the practical course and/or the work experience programme at Tomaree.

During 2004 there were more than 14 tours of the facility from indigenous groups (and several others from non indigenous groups) involving more than 75 Indigenous people. During these tours the facilities were inspected and one of the staff gave an introduction to land based flow-through aquaculture. After which a more detailed explanation of the basics of abalone culture was given. The tours of the abalone facility were in most cases followed by a tour of the Port Stephens Fisheries Centre. Here introductions were given to oyster and finfish aquaculture.

In late 2003 and early 2004 several meetings with representatives of Hunter TAFE took place to tailor the Tomaree practical training programme to the needs of NSW TAFE students. A total of 8 training courses were staged for coastal indigenous community groups and students enrolled in an aquaculture induction training courses staged at the Tomaree and Wollongong colleges of TAFE. An outline of this induction training course was produced and vetted by the Tomaree TAFE and the IFS working group. Three topic areas were identified as important and incorporated into the training plan: routine and maintenance skills; aquaculture construction experience; and shellfish breeding and larval husbandry.

To further assist indigenous groups on the south coast of NSW in their quest towards sustainable farming and or fisheries enhancement and ranching of blacklip abalone, a practical guide to commercial scale hatchery production has been produced. This stand alone publication provides comprehensive step by step instruction on how to collect and reproductively condition blacklip abalone broodstock, how to induce them to spawn and how to hatchery rear their young though larval and early juvenile (spat) stages to an age and size suitable for on-farming or for seeding depleted fisheries. In view of the scarcity and high cost of coastal sites with access to marine waters in NSW, the techniques described are geared to efficient and intensive production of abalone spat using a small area of land. These techniques in some respects are quite different to those generally practiced to date by commercial abalone farms in Australia.

5.4.   Objective 4: Develop a bio-economic model for cost-effective fisheries enhancement of New South Wales blacklip abalone, *Haliotis rubra* (Leach) fishery

The major benefit of this model is that it has focused limited R&D resources towards the type of seed ("button size" juveniles) and seeding methods (low density highly dispersed) seeding employing predator protective devices deployed from surface craft) that offer best inherent prospects of being cost-effective.

6.   FURTHER DEVELOPMENT

6.1.   <u>Objective 1</u>: Produce and assess triploid *H. rubra* for farming and fisheries enhancement

A provisional patent for technology relating to a specific method of triploidy induction that could double yields of saleable foot flesh in 3.6 year old *H. rubra* was registered by the FRDC and NSW DPI in April 2007. FRDC is currently (May 2007) negotiating a licensing agreement with a large Victorian based abalone farming corporation to further assess and commercialise this technology in relation to *H. rubra*, *H. laevigata* and hybrids thereof on behalf of the wider industry throughout southern Australia.

6.2.   <u>Objective 2</u>: Evaluate alternative methods of reducing high post-release mortality rates commonly sustained by hatchery produced abalone

A large-scale long term seeding experiment initiated in July 2005. Six seeded and six non seeded control sites will be resurveyed in late 2008 or early 2009 to assess survival through to market size of seeded juveniles together with possible impacts of seeding on the reef plant and animal communities. This will be followed up with subsequent surveys in 2012 and 2015 to gauge long term genetic impacts on wild stocks.

6.3.   <u>Objective 3</u>: Develop indigenous community capacity to undertake the production of seed abalone and use of that seed for cost effective and sustainable fisheries enhancement, ranching and farming of back-lip abalone

Further developments are subject to on going discussion and negotiations between NSW Government and south coast indigenous groups.

6.4.   <u>Objective 4</u>: Develop a bio-economic model for cost-effective fisheries enhancement of New South Wales blacklip abalone, *Haliotis rubra* (Leach) fishery

An experiment to input data into a bio economic model for enhancing depleted *H. rubra* stocks with hatchery produced "button size" juveniles was initiated on the Tomaree Peninsula, Port Stephens in August 2005. Commercially significant numbers of "button size" juveniles were experimentally seeded onto six 1000 m^2 experimental sites using specially developed low density dispersed techniques aligned to the carrying capacity of juvenile habitats and ultimately to those of adjacent adult habitats. A resurvey of seeded and an equal number of control non seeded sites in will be conducted in late 2008 or early 2009 to gauge long term survival of released hatchery seed and hence the likely cost-effectiveness of these techniques.

7.   PLANNED OUTCOMES

(1)   Facilitated establishment of one or more abalone hatcheries in NSW incorporating leading edge technology involving at least one indigenous (total or majority) owned abalone hatchery business in southern NSW.

(2)   Capacity in one or more indigenous groups to utilise the abalone technology both at community and business level thereby generating significant additional employment and income for coastal indigenous Australians.

(3)   Cost-effective fisheries enhancement, and/or ranching and/or offshore farming of abalone in southern NSW levels particularly through enhancement of abalone stocks in areas important (food and culturally) to coastal indigenous Australians.

(4)   Achievement of high yielding fisheries enhancement and ranching of abalone operations that do not pose significant long-term or widespread environmental risks.

(5)   Production of an interactive economic model for blacklip abalone fisheries enhancement and ranching available on CD for interested stakeholders and private sector investors.

7.1.   Actual outcomes

(1)   Despite technical advice, an approval process facilitated by NSW DPI Aquaculture Management and a generous subsidy offered through the NSW Government Indigenous Fisheries Strategy, a suitable proposal for an abalone hatchery on the NSW south coast was not developed. There is ongoing interest in abalone and other aquatic species among indigenous communities and if business planning and investment analysis for these operations matures and indicates promise, it is still possible that an indigenous abalone/shellfish hatchery will be constructed.

(2)   Capacity for indigenous groups has been increased through targeted training programs and the development of a comprehensive abalone production manual. Additional training opportunities through NSW TAFE for indigenous Australians are ongoing and technology developed during this project and made available to TAFE staff, will ensure new trainees can benefit from this project.

(3 & 4)   Not achieved at this stage although if positive results for the large-scale experiment sill in progress are received this is likely to stimulate renewed interest in enhancement. A policy for offshore ranching is still under development in NSW. No offshore farms have been proposed in NSW although the technology and results developed during this project will assist any investors interested in such an operation.

(5)   The bioeconomic model has been developed and a CD will be completed when final data for the large-scale seeding experiment is available in 2009.

8.   CONCLUSIONS

8.1.   Objective 1: Produce and assess triploid *H. rubra* for farming and fisheries enhancement

Optimised methods to induce triploidy in *H. rubra* using the chemicals 6-DMAP and Cytochalasin B have been successfully developed. Those employing 8 DMAP have been shown to be superior yielding higher quantities of viable stock that exhibit higher rates of triploidy (at or near 100%). These same stock are reproductively sterile which renders them low risk candidates for enhancing depleted *H. rubra* fisheries stocks in NSW and elsewhere in Southern Australia. They have also been shown to exhibit potentially significant advantages over normal diploid counterparts in terms of growth rate and yield of marketable foot muscle flesh at 3.6 years of age. This technology has received IP protection and is attracting considerable interest from industry.

8.2.   Objective 2: Evaluate alternative methods of reducing high post-release mortality rates commonly sustained by hatchery produced abalone

Reducing seeding rates from 1000 to 100/m^2 does not appear to solve the problem of high post release mortality of hatchery produced "button size" abalone. Unusual shell colour of hatchery produced seed does not appear to jeopardise post release survival. Releasing juveniles under and around the common purple urchins does not enhance survival. Results of a very large experiment to test the utility of low density (10/m^2) dispersed seeding for achieving cost-effective enhancement of depleted *H. rubra* populations will not be known for a further 18 months or so (2009). Assessment of genetic impacts of such seedings will not be possible for at least a further 3 years (2012).

8.3.   Objective 3: Develop indigenous community capacity to undertake the production of seed abalone and use of that seed for cost effective and sustainable fisheries enhancement, ranching and farming of back-lip abalone

Achievement of cost-effective land based farming and fisheries enhancement of abalone in Australia is proving more problematic than originally anticipated by industry at the outset of this project in 2001. Accordingly further investment in abalone aquaculture and fisheries enhancement training programs for the benefit of indigenous groups in southern NSW is not recommended for the time being.

8.4.   Objective 4: Develop a bio-economic model for cost-effective fisheries enhancement of New South Wales blacklip abalone, *Haliotis rubra* (Leach) fishery

Critical input data for this model are subject to completion of ongoing research in about 18 months time. Key factors affecting the accuracy of this model in predicting cost-effectiveness of enhancing depleted abalone fishery in NSW are age and size specific growth and natural mortality rates and carrying capacity crustose coralline coated rock habitats of surface grazing juveniles. Fisheries production limitations for *H. rubra* also encompass the influence of other species within reef ecosystems. Especially important are predators such as wrasse plus and other large surface grazing gastropods and urchins. In NSW urchins that outnumber abalone by at least 2 orders of magnitude not only compete directly for food (epiphytes and drift seaweed), space and shelter, but may also limit *H. rubra* recruitment by inadvertent grazing of recently settled postlarvae.

9. APPENDICES

9.1. Intellectual property

The intellectual property associated with the abalone triploidy is vested with FRDC. All other information brought into this project or developed during the project is in the public domain.

9.2. Staff

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9.3. Publications

Dworjanyn S., Pirozzi, I. and Heasman, M.P. 2003. Hybridisation between blacklip abalone (*Haliotis rubra*) and the scarlet rayed abalone (*Haliotis coccoradiata*). In: *Proceedings of the 10th Annual Abalone Aquaculture Workshop, 19 – 21 November 2003, Port Lincoln, South Australia.* Fleming, A.E. (Editor). Fisheries Research and Development Corporation's Abalone Aquaculture Subprogram, Canberra, Australia.

Heasman, M.P. and Savva, N. (In press). Manual for Intensive Hatchery Production of Abalone: Theory and practise for year-round, high density seed production of Blacklip Abalone (*Haliotis rubra*). NSW Department of Primary Industries.

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Liu, W., Heasman, M.P. and Simpson, R. 2004. Evaluation of cytochalasin B (CB) treatments for triploidy induction in the blacklip abalone, *Haliotis rubra* (Leach, 1814). *Aquaculture Research* 35, 1062–1075.

9.4. New technology for enhancing the abalone fishery in NSW, Australia (poster)

9.5. **Sea Urchin (*Tripneustes gratilla*) Hatchery Manual**

CULTURING SEA URCHIN LARVAE

A Manual for Raising the Larvae of *Tripneustes gratilla*

Symon Dworjanyn

2007

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Chapter

1

Introduction

The Market and Fishery

Sea urchins are a traditional food in many cultures around the world including in North and Southeast Asia, the Mediterranean, South America and the Pacific. Japan is by far the largest market for sea urchin, consuming more than 80% of the annual world commercial catch. The wholesale value of this market is estimated to be between 300 and 600 million USD$. Almost all of the current world production of sea urchin is from wild caught fisheries. World production peaked in 1995 at 120 000 t and since then has been in decline. Many individual fisheries have been in decline well before the mid 1990's and much of the present production of sea urchin has been made possible by exploitation of previously virgin stocks. Currently, the majority of sea urchin roe is produced in the USA, Chile South Korea and Canada. The gonads of both male and female sea urchins are eaten and are collectively termed roe. In Japan sea urchin roe called *uni* (Figure 1), is usually eaten raw by itself (sashimi) or with rice (sushi). The Japanese market demands brightly coloured yellow to orange roe that is about 4cm long, firm and sweet with no bitter aftertaste.

Fig 1. Sea urchin roe (Uni) ready for market.

Sea Urchin Aquaculture

The sharp decline in wild sea urchin fisheries has created interest in sea urchin cultivation. Cultivation of sea urchins has taken two approaches; enhancement of wild caught animals and closed culture of animals from spawning through to harvest.

Enhancement

Fig 2. Opened sea urchin (*Tripneustes gratilla*) with roe in place.

The quality and quantity of food a sea urchin has access to will determine the value of its roe. Often wild harvested animals caught from areas where there is a low standing stock of algae resulting in small, low quality roe that receive poor prices or are unacceptable to the market. Post harvest enhancement of roe has been touted as a way of in proving the profitability of such fisheries. Sea urchins are opportunistic feeders, quickly taking advantage of food when it becomes available and directing much of this food to reproductive output. This lends them to quick growth of roe under culture conditions. Under experimental conditions the size of gonads of wild caught sea urchins across a range of species has consistently been enhanced from about 3 – 7% to about 15 – 20%. This is achieved by feeding the sea urchins a diet of mixed seaweeds or artificial diets with a moderately high protein level. Typically, in these experiments increases in gonad size peak at between six and ten weeks of feeding on the high quality diet. One of the early problems faced with this approach was that artificially enhanced gonads were often dull in colour. This has largely been solved by the addition of carotenoids to artificial diets. Carotenoids are compounds most commonly found in orange coloured vegetables. Carotenoids are only effective however if they are from a natural source. A good source of natural carotenoids is produced in Australia by extracting microscopic algae (Algro™, Cognis Australia). Further problems with artificially enhancing gonad size include achieving desirable taste and firmness. These problems are currently being investigated at several research institutions around the world. Currently, post harvest gonad enhancement is not being used on a commercial scale.

Closed Culture

The first attempts at semi-closed culture of sea urchins were done in Japan to supply juvenile animals for release into the wild to bolster dwindling wild stocks. This started in the late 1970's and in 1996, 78 464 million hatchery reared sea urchins were released into Japanese waters. This programme continues today. More recently, attempts have been made to develop systems for growing urchins from spawning to harvest. To date the only commercial urchin farms that we are aware of are in China however, data on their output are not available. The Chinese farms and Japanese reseeding programmes both feed the juvenile and adult animals seaweed which is unlikely to be feasible in a commercial Australian operation. There are several experimental projects in North America and Europe looking at closed culture as well as the release of hatchery reared animals to bolster overfished wild populations but they all are currently in the development stage.

What Species are of Economic Importance in Australia?

Australia is the home of many sea urchin species that are often the dominant organism shaping subtidal habitats. For example, *Centrostephanus rodgersii* (a large, black urchin) is a keystone species responsible for determining the distribution of kelp and other fleshy algae on reefs in the temperate east coast of Australia. Despite the large numbers of sea urchins in Australia they support very small fisheries restricted to *Heliocidaris erythrogramma* in Tasmania and *H. tuberculata* in NSW. Combined, the annual landing of these two fisheries is probably less than 300t. Some of

Fig 3. *Tripneustes gratilla.*

the reasons for the small sea urchin fishery in Australia include the poor gonad yield and the poor quality of roe from Australian sea urchins. The slow growth of these two species will most likely preclude them from intensive aquaculture except possibly for post-harvest enhancement of gonads.

There is another species in Australia that has a tropical/subtropical distribution that shows promise for aquaculture. *Tripneustes gratilla* (Figure 3) is found throughout the tropical Pacific including southern Japan. It is one of the most valuable species of sea urchin on the Japanese market where it is called *baffun*. It is common on Australia's east coast but its patchy and unpredictable distribution has precluded a commercial fishery. The value of this species as an aquaculture species lies in its fast growth. Possibly because it's a tropical sea urchin *T. gratilla*, reaches maturity in less than 12 months in the wild. Most other species that have been assessed for they suitability for aquaculture around the world are from temperate waters and have slow growth rates not reaching maturity for 2 – 4 years. In Australia *T. gratilla* is found as far south as Merimbula and shows fast growth rates even during winter in Sydney waters. The rearing instructions in this manual are based on this species.

Biology and Anatomy

Sea urchins belong to a group of animals called the echinoderms that also include sea stars, brittle stars and sea cucumbers. Echinoderms are unique among the invertebrates in that they have an internal skeleton made from calcium carbonate, which in sea urchins is called a test. Echinoderms are also particular in that they have a water vascular system in which water in a series of tubes transports metabolites and waste around their bodies. Sea urchins have a five-way symmetry with body structures (eg. gonads) repeating five times in a radial arrangement. Sea urchins are covered by hard spines that are controlled by muscles and are used for movement and for protection from predators. Tube feet extending from their body are controlled by the water vascular system, and are used for locomotion, smell and taste, to grip food and adhere to surfaces. The anus and the gonadopores (where sperm and eggs are released) are located on the top of the body. The mouth of the sea urchin, made of five pincer like teeth is on its bottom surface. Sea urchins are mainly herbivores meaning they eat plants, in the wild this is usually seaweeds.

The Life Cycle of Sea Urchins

Sea urchins are broadcast spawners releasing their eggs and sperm into the water column to be fertilised. The fertilised eggs hatch into larvae that swim in the water column. Some species of sea urchin larvae have a yolk that they feed on and only spend a short time in the water column (1 – 2 weeks) but most species feed on single celled algae and spend extended periods floating in the plankton. The time spent as larvae varies among species and is influenced by the availability of food and water temperature. As a general rule, larvae develop faster in warmer waters. During their development larvae go through several changes from the blastula stage through to the many armed plueteus stages. When larvae are ready to metamorphose into the adult form they are said to be competent. Competent sea urchin larvae are thought to search for habitats that will increase their chances of surviving and reproducing when they find these habitats they metamorphose into a form that looks the same as the adult. They find these sites by detecting chemicals dissolved in the water column released by organisms associated with desirable habitats. Sources of these chemical cues include several species of seaweed, bacteria, diatoms. At first juvenile sea urchins are too small to eat seaweed and gain nutriment by eating diatoms and bacteria that they scrape from surfaces. Once their gut and mouthparts are sufficiently developed they start eating their adult diet of seaweed. Time to maturity can range from under a year to many years depending on species and environmental conditions.

Before you start

Hygiene

Hygiene is very important when rearing any larvae. Good cleaning practices reduce the probability of disease and increase success rates. All equipment that is used in spawning or rearing larvae needs to be cleaned before use. A good way to do this is to use a chlorine dip.

Fig 4. Water heating unit fashioned from a plastic drum with two thermostatically controlled heaters, a float valve and outflow tap (controlled by a pump).

Fill a 60l fish box with about 5% pool chlorine in fresh water. Then make a second rinse bath with only fresh water. All pieces of equipment should be dipped in these baths before they come in contact with larvae. Equipment can be left soaking in the chlorine solution and rinsed in the freshwater bath immediately prior to use or equipment can be dipped the chlorine bath, then the fresh water bath, and placed on a rack to dry. All airlines and water lines should also be cleaned and sterilised regularly by dipping in the chlorine bath. Larger pieces of equipment can be rinsed with the chlorine solution and then hosed down with freshwater. It is important to note that although chlorine is a good sterilising agent it will kill larvae if they are exposed to it so it is crucial that equipment is well rinsed of chlorine before coming in contact with larvae.

Equipment

There are many different ways to conform equipment to do the procedures described here. Below is a brief description of some of the equipment that we use for rearing sea urchin larvae at NSW Department of Primary Industries. Much of the equipment we use is built from scratch or modified by us to suit our needs.

Tripneustes gratilla is in the main a tropical species and their larvae grow best in warmer water – we suggest 25°C. If your water supply is cool it will have to be warmed. A good way to do this is to maintain the larvae in a constant temperature room set at 25°C. At NSW DPI, constant temperature rooms are fabricated from insulated shipping containers with reverse cycle air-conditioners installed. Constant temperature rooms also need to be fitted with air, fresh and seawater supplies. To initially heat the larval rearing water up to 25°C we use a 125L seawater reservoir with two thermostatically controlled heating units attached (Figure 4).

Larvae are reared in round fibreglass containers with conical bases (Figure 5). At the bottom of the containers we fit the air supply that is controlled with a screw clamp (Figure 6). The air supply can be removed and this port can be used to drain the tank for harvesting or tank changes. The conical bottom allows complete mixing and aeration of the larvae with no dead spots when air is supplied from the base. A 125l containers work well however smaller or larger containers can be used as suit your needs. A banjo screen is fitted to the overflow of the rearing container (Fig 7) so that water can be changed without losing larvae. The screen size has to be smaller than the larvae however, if it is too small it will get clogged to often, about 70μm is good.

Fig 5. Two fibreglass larval rearing vessels on stands, showing overflow tube and top of the banjo screen (protruding from the banjo screens are thermometers).

General laboratory equipment is also needed including beakers of various sizes, pipettes, measuring cylinders, a balance, and storage bottles. A good compound microscope is essential to inspect the eggs, sperm and larvae Sedgewick Rafter slide and a hemocytometer both of

which are described below are needed for use with the microscope. A dissecting microscope is also useful but not necessary.

Fig 6. Outlet of the rearing vessel showing the air inlet controlled by a clamp that can also be used as a water outlet for draining the tank.

Depending on how you settle your larvae you may need to grow diatom biofilms. To grow a biofilm you will need outdoor tanks that are exposed to sunlight and have a constant supply of seawater. These tanks will also need to be supplied with ample aeration supply. The design of these tanks are not crucial as long as they can fit settlement plates an example of which is described in detail below.

Fig 7. Banjo screen fitted to the over flow of a larval rearing vessel (note the conical bottom of the vessel).

Water

All of the seawater we use for spawning and rearing sea urchin larvae is pumped from an oceanic site and passed through an 18μm drum filter. It is then run through a bank of three cartridge filters with nominal sizes of 10μm, 1μm and 1μm and finally through a UV sterilising unit (Figure 8). In the rest of the manual we refer to the water that has been treated in this way as filtered seawater. When the water lines in the hatchery are not in use they are disconnected and allowed to dry so that there is no residual water allowed to sit in any of the lines or filtration equipment. The filter cartridges are washed and rinsed in the chlorine solution daily, taking care to rinse all residual chlorine from them before they are used again. The configuration of pumps, air blowers, outside tanks etc. will largely be constrained by your site, budget and needs and is outside the scope of this manual.

Fig 8. Three cartridge filters and the UV sterilisation unit in line with the seawater supply hose.

REARING LARVAE

Obtaining Broodstock

Broodstock can be collected from the wild but be sure to find out the regulations regarding collecting sea urchins for aquaculture in your state before proceeding. In NSW, *Tripneustes gratilla* has a patchy distribution over both time and space so can be hard to find. Moreover, a site that you collect them from one season may have none the next season. The reason for this patchy distribution in southern populations may be because they depend on larvae transported in currents from more tropical waters in the North. A good way to find *T. gratilla* is to ask local dive operators. This task will be easier if you have a good quality photograph of the urchin. *T. gratilla* in NSW appear to have a defined spawning season. Although the data for this is patchy and inconsistent it seems that the best time to collect them from the wild to spawn is in late summer and early autumn. When kept in culture and fed high quality feed it might be possible to extend the spawning season. The spawning season for *T. gratilla* in other regions vary greatly and there appears to be no general predictive environmental cue that relates to gonad development. The quality of a sea urchins gonad is strongly dependant on food it eats. Because of this if sea urchins are collected from large dense aggregations where there is little seaweed they will have small gonads that may be difficult to spawn and produce low numbers of eggs and sperm. If these are the only animals you have access to a month or more fed a mixed macroalgae diet might improve their reproductive output.

Tripneustes gratilla is found in the sub-tidal in depths down to 75 m so in most cases their collection will be done on SCUBA. They are found in a range of habitats but prefer more sheltered sites and are rare on high-energy reefs. Their habit of covering their body with pieces of

Fig 9. Injecting 1ml KCl into the interceolonic membrane of *Tripneustes gratilla.*

detritus and seaweed make them cryptic in the wild. Sea urchins are delicate and easily damaged, when collecting or transferring them between containers it important that that they are removed from surfaces as gently as possible to avoid breaking tube feet. Do not expose the sea urchins to air for anything but very short periods of time. Sea urchins should be transport in fresh seawater that is supplied with ample oxygen or air and maintained at a similar temperature to the environment that they were collected from. The practice of transporting sea urchins under wet sacks as done by commercial divers is not desirable as it can induce spawning. For collecting small numbers of sea urchins a bucket of seawater supplied with air in an air conditioned car might suffice however, the more urchins to be moved the more elaborate your transport equipment will have to be. Once at your hatchery the sea urchins should be supplied with ample flowing seawater and a diet of mixed seaweeds. The kelp *Ecklonia radiata* and the rockweed *Sargassum* sp. are plentiful, easily collected and provide a good diet for *T. gratilla*.

Spawning

Spawning sea urchins is easy and needs little specialised equipment. First, make a solution of 2 molar potassium chloride (2M KCl), by mixing 149g of potassium chloride in 1 litre of fresh water. Place the sea urchin mouth side up on a 200ml beaker filled with fresh filtered seawater. Inject 0.5 – 1ml of the 2M KCl in to the body cavity of the sea urchin through the soft membrane that surrounds the mouth (Fig 9) using a hypodermic needle and syringe. After about a minute the sea urchin should start releasing eggs or sperm as a stream from the top of the animal into the water. It is impossible to sex sea urchins using external features. The sperm released in a series off-white creamy streams (fig 10). The eggs are slightly darker to orange and come out in a more discontinuous flow (Fig 11). Both the sperm and eggs will remain viable for several hours. Each female should produce many millions of eggs and a male will produce sperm well in excess of that need to fertilise many females. To create some genetic diversity in your stock several females and males should be used at each spawning.

Fertilising eggs

To fertilise the eggs you should aim to add about 20 sperm for every egg. Too few sperm and not all of the eggs will be fertilised too many sperm and they will break down the protective layer around the egg and kill it. With practise you will be able to judge how much sperm to add to eggs by eye but at first it is best to get a more accurate egg and sperm count. To count the eggs, pool all of the eggs in one container. Mix the eggs gently until you have a homogenous solution then take a small sample of this solution of known volume (about 1ml). Place this sample on a white background, for example a weighing boat, to provide a contrast with the dark eggs. Count the number of eggs in the sample. The eggs are about a tenth of a millimetre in diameter and are visible to the naked eye. If there are too many eggs in your sample to count easily dilute your egg solution with seawater or take a smaller sample volume. Repeat this three times and get an average in your sample. Multiply this number by the volume of you entire egg solution and you will have an estimate of the number of eggs you have. (For example: if you have an average of 500 eggs in each 0.25 ml sample and a litre of egg solution you have approximately 2 000 000 eggs i.e., number of eggs per ml: $500 \times 4 = 2000$. The number of eggs in total: $2000 \times 1000 = 2\ 000\ 000$).

Fig 10. A male *Tripneustes gratilla* releasing sperm in streams from the gonadopores that collects at the bottom of the container.

Fig 11. Water heating unit fashioned from a plastic drum with two thermostatically controlled heaters, a float valve and outflow tap (control by a pump).

To count the sperm you will need a hemocytometer used to count blood cells. The hemocytometer consists of a thick glass slide that fits on a microscope stage and a smaller cover slip that sits on top of the glass slide. On the surface of the glass slide is etched a series of grids. By counting (using a microscope) the number of cells (or sperm) in each of these grids you can get an estimate of the density of cells the solution. To get an estimate of your sperm concentration, first mix some of the sperm from each of the males in a beaker (you will not need all of the sperm). Spermatozoa are very small and should be very active. Take a few millilitres of the sperm solution and add a drop or two of the chlorine solution that you use to

was your hatchery equipment. This will kill them to stop them moving. Take a drop of the dead sperm solution and placed it on the hemocytometer and place the cover slip placed on top of this. To get an estimate of the sperm density you will have count the number of sperm in several of the grids on the slide, to convert these counts to densities follow the conversion instructions that come with the hemocytometer.

Once you have estimates of the number of eggs and the density of sperm you have add enough sperm to your eggs so that there are about 20 sperm for each egg (for example if you have 2 million eggs and 5 million sperm per ml you will have to add 4ml of sperm solution to the eggs). Before adding the sperm to the eggs look at a sample of the sperm down the microscope to ensure that the sperm are active. If the sperm are not moving they are dead and will not fertilise the eggs. Add the appropriate amount of sperm to the eggs stir the solution gently. Wait a few minutes and inspect the eggs under the microscope. There should be able to see about ten sperm attached to each egg (Figure 12). If there are no sperm attached to the eggs add more sperm. If there are sufficient sperm attached to the eggs or too many you next wash the eggs.
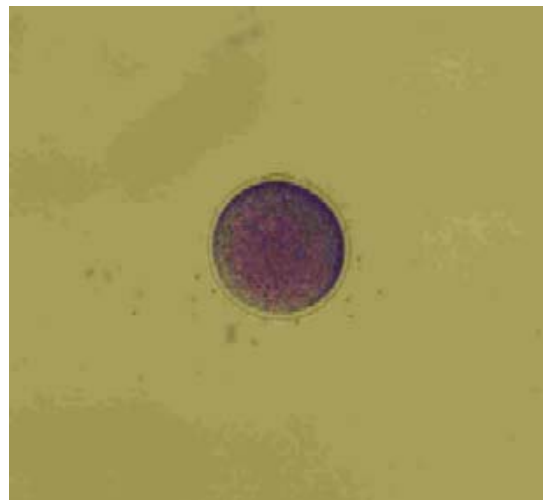

Fig 12. Sperm attach to a sea urchin egg.

Once fertilised the eggs are washed in a wet sieve. A wet sieve consists of a sieve that is placed in a container filled with water so that the sides of the sieve are above the water level but the mesh of the sieve is always under water. This allows delicate things like eggs and larvae to be sieved without damage (note: urchin larvae are so delicate that even a wet sieve will often damage them). Place the fertilised eggs in the wet sieve and gently flow filtered seawater over them. The eggs will be just under 100μm in diameter so the screen should be 70 μm or less. This step removes any excess sperm and extraneous material that is released with the eggs. The eggs are now ready for the larval rearing vessel.

Rearing

Sea urchin larvae are very delicate and need to be carefully handled. We rear the larvae in 125l round container with a conical bottom as described above. Water temperature is maintained at 25°C – below 20°C the larvae will not develop to competency. The fertilised eggs are placed in the rearing container with gentle aeration. Eggs hatch after approximately 24h but do not begin to feed until 2 to 3 days after fertilisation. Once hatched the density of larvae should be reduced to about 10 per ml. Over the life of the larvae this density is slowly reduced through mortalities and losses that occur during tank changes. The larvae are competent after approximately 35 days by which time their density should be reduced to about 4/ml.

Feeding

Tripneustes gratilla larvae feed on single celled algae floating in the water column. In culture they grow well on the pelagic diatom *Chetocerous meullerii.* (The production of algae to feed larvae is out of the scope of this manual.) Start feeding the larvae two days after fertilisation, not all of the larvae will be ready to feed at this point however, this will ensure that food is available as soon as they start feeding. Initially, feed the larvae at a rate of 4000 cells of algae per larvae. To work out feeding rates first work out the density of algal cells in your culture. This is done the same way you worked out sperm densities during fertilisation except the diatoms not need to be killed before they are placed hemocytometer. Next work out your larval density. The gentle aeration applied to the larvae should evenly distribute them throughout the culture vessel. Using a pipette take a one millilitre sample and place it on a Sedgwick Rafter slide (Fig 13). Count the number of larvae in your sample under a compound microscope. Repeat this several times and calculate the mean in your samples. Multiply this result by the volume of the culture vessel to get an estimate of how many larvae you have. To work out how many millilitres of algae to use multiply the number of larvae you have by the number of cells you intend to feed per larvae and then divide this number by the density of cells in your larval culture. Be careful when adding food, if you add the food directly you will lose this volume of water from the overflow. To overcome this, reduce the water volume in your tank by displacing a volume equal to the volume of food you are going to add.

Fig 13. Sedgewick rafter style slide used for counting larvae.

As the larvae grow they progressively eat more. The week prior to competence they should be fed about 10 –15 000 cells per larvae per day. To assess when to increase your feeding rate, observe the larvae before feeding each day the stomach should be full. If it is empty you are not feeding the larvae enough. Alternately, before cleaning the culture vessel each day do a count of the number algal cells left in the culture. If the larvae are clearing approximately 70% or more of the cells each day you should increase your feed rate.

Daily maintenance

As larvae are stocked at high densities and large numbers of diatoms used in daily feeding; water quality becomes a major concern. To maintain good water quality we recommend that a quantity of filtered seawater equal to two volumes of your rearing container is exchanged daily. To do this first fill your heating reservoir with filtered water and heat it to 25°C. Pump this water into the larval rearing container at a flow rate of about $1 - 2$ L/min; faster flow rates will damage larvae by trapping them on the banjo screen. The excess water should drain out the over-flow and the banjo filter will prevent the loss of larvae. Ensure that aeration is maintained while doing this. Check the banjo screen daily and clean it if there is build-up of matter.

Each day keep records of your larval culture in a laboratory notebook. Record larval numbers, feed rates, feed clearance rates, water temperature and general comments about the larvae for example, a quick sketch describing their development stage and a note as too whether their guts are full. This sort of record keeping allows you to pinpoint problems and solve problems before they become serious.

Weekly maintenance

Daily water exchanges ensure good water quality. However, biofilms and debris will still accumulate on the tank walls over time. Therefore, weekly tank changes are required to maintain good hygiene. To do this first perform the daily water exchange to remove uneaten diatoms and other suspended particles from your culture. As urchin larvae are very fragile screening as would be done with other types of larvae should be avoided where possible. Partially fill the new tank with 25°C, filtered seawater and transfer the larvae by gently bucketing them from the old container. Using this method there will be some water and larvae left behind, this can be discarded, as it will also be mixed with waste and old food. There will be an incremental reduction in larval densities in your culture using this method that compliments the increase in feeding rates of larvae as they develop.

Rafting larvae

As larvae develop they become larger and have a more complex shape. This can cause a phenomenon where the larvae trap air bubbles on their bodies and float to the surface of the water stop feeding and die. This is a serious problem that can kill your whole culture in as little as a day. A quick fix when this happens is to change the water tension in your rearing container by the addition of acetic alcohol at a rate of # per litre. However, in the longer term the cause of the problem should be fixed. It is usually caused by the production of very small air bubbles in your culture water that are usually caused by a pump that is sucking in air. A once over of all the pumps and air systems can usually identify the culprit.

Settling larvae

To induce metamorphosis in sea urchin larvae they have to be exposed to a chemical cue. In the wild it is thought that the larvae use chemical cues to find habitats that are favourable for growth and reproduction. There are several cues that induce metamorphosis of *Tripneustes gratilla* larvae including several species of seaweed, diatom biofilms and adult conspecific. We suggest a good cue to use in a hatchery is a diatom biofilm. A biofilm is a thin film of bacteria and plants like diatoms that grow on any surface immersed in seawater. Biofilms have the

added advantage that as well as inducing settlement they provide a source of nutriment newly settled sea urchins.

Tripnuestes gratilla have a long larval phase. Under the conditions described here larvae are competent to settle after about 35 days. The length of the larval stage results in a large spread of development in cultured larvae and this can cause difficulties in assessing when to settle your larvae. We suggest you settle your larvae when greater than fifty percent of the larvae are competent. How do you know if the larvae are competent? One way is to inspect the morphology of the larvae. Larvae should be competent to settle when the rudiment is larger than the gut (Figure 14). Another way is to assess competency is to do a settlement assay. To do this, fill five petri dishes (most types will do) with filtered seawater. Into the petri dishes scrape a couple of square centimetres of diatom biofilm and place 10 – 20 larvae to into each dish. Maintain the petri dishes at 25ºC. After 48 h count the average percentage of larvae that have metamorphosed (Fig 15). if 20% or more have metamorphosed it is a good time to start settling them.

Fig 14. An eight arm pleuteus *Tripneustes gratilla* larvae close to competence. The structure on the left of the gut is the rudiment.

One way to settle large numbers of larvae is to use a system similar to that used by abalone farmers. In most hatcheries in Australia abalone are settled onto Perspex plates that are 600mm x 300mm and 3mm thick to which a diatom biofilm is grown. These plates are held in plastic coated wire baskets that hold 16 plates vertically on their long side. To grow a biofilm the baskets are held fully submerged in partially shaded outdoor tanks, typically eight baskets to the tank. The tanks are supplied with flow-through seawater and ample aeration under the baskets. It is a good idea to run the water entering a settlement tank through a 10 µm cartridge filter to limit the entry of epiphytes other than diatoms from entering the settlement tank. A biofilm appears as a thin brown film on the surface of the plate. The growth rate of diatoms on settlement plates will depend on water temperature, light, oxygen and the nutrients in the water. By manipulating these factors the growth of diatoms and other algae on settlement plates can be increased or decreased. The speed of biofilm development also varies with time of the year and even among settlement tanks in a hatchery. In general, it takes about three weeks to grow a good biofilm on settlement plates during summer.

Fig 15. A newly metamorphosed *Tripneustes gratilla* juvenile.

To settle the larvae first turn off the seawater flow to the tank holding the settlement plates and maintain gentle aeration beneath the settlement plates. Although sea urchin larvae are very delicate by the time they are competent they are robust enough to withstand being wet sieved. If you choose you can harvest you larvae by setting up a wet sieve and very slowly draining the contents of the larval rearing tank over the sieve. These larvae can then be resuspended in a smaller volume of water and placed evenly in your settlement tank.

Alternately, the larvae can be directly removed from the rearing container using a siphon or a bucket and the larvae placed directly into the settlement tank. Water flow into the settlement tanks should remain off for at least two days to allow the larvae to metamorphose. Once metamorphosed over five to seven days the larvae will develop a mouth-parts and a gut that is the same as the adult. Some references say the newly metamorphosed larvae can only feed on diatom biofilms for some weeks after metamorphosis others maintain that as soon as their mouthparts are developed they can begin to eat some of the less tough macroalgae such as *Ulva lactuca.*

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