Triploid Bay Scallops (*Argopecten irradians*): Induction Methodology, Early Gonadic Development and Growth

A.T. Cogswell, E.L. Kenchington, S.E. Roach and B.W. MacDonald

Science Branch
Maritimes Region
Ecosystem Research Division
Department of Fisheries and Oceans
PO Box 1006
Halifax, Nova Scotia
B2Y 4A2

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A.T. Cogswell, E.L. Kenchington, S.E. Roach and B.W. MacDonald

Science Branch, Maritimes Region
Ecosystem Research Division
Fisheries and Oceans Canada
1 Challenger Drive
Halifax, NS
B2Y 4A2
E-mail: CogswellA@mar.dfo-mpo.gc.ca

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ABSTRACT

Cogswell, A.T., E.L. Kenchington, S.E. Roach and B.W. MacDonald. 2006. Triploid bay scallops (*Argopecten irradians*): induction methodology, early gonadic development and growth. Can. Tech. Rep. Fish. Aquat. Sci. 2635: v + 48 p.

Triploid (3N) Pacific oysters (*Crassostrea gigas*) account for more than 50% of total oyster production in the US. The success of this species has created an interest in producing triploids of other commercial shellfish species, including scallops, clams and mussels. Here, we report on 3N induction trials with the bay scallop, *Argopecten irradians*. The most commonly employed 3N induction technique involves exposing early embryos to chemicals (e.g., Cytochalasin B (CB)). CB inhibits the release of the second polar body immediately following fertilization, causing retention of both pairs of female chromosomes in addition to the male chromosome set. It does this by disrupting actin polymerization. Four concentrations of CB were evaluated for ability to produce 3N larvae. Concentrations of 0.1, 0.5 and 0.75 mg/l, resulted in ~25%, ~40% and ~60% 3N D-larvae at day 4 respectively. In commercial production 80% induction is deemed successful. This was achieved at a CB concentration of 1 mg/l which produced 97-100% 3N D-larvae at day 4. These later larvae were monitored over 18 months from juvenile to adult, and induction rates did not noticeably decline. There was no difference between the 3N and control (2N) larval growth between 6 and 13 days post-spawn. Triploid and control bay scallops transplanted to an aquaculture site exhibited no significant differences in shell dimensions after 3 months but the 3N group showed a significant 8% increase in muscle indices ((dry muscle weight/total tissue dry weight)*100) offset by an inversely proportional 6% decline in gonad indices as compared to diploids (2N). This is evidence that 3Ns uniquely partition energy reserves. Reproductive development was severely stunted in 3Ns during the peak 2N spawning period. The 3N animals remained hermaphroditic, although histological examination of the gonads showed that egg size was significantly larger (~1.2x) than in 2Ns and sperm development was halted at the primary spermatocyte stage. In addition, egg fecundity was drastically reduced in 3Ns and the small egg follicles appear to be inundated with phagocytic haemocytes suggesting poor follicle health and continual oocyte reabsorption. The efficacy of current and proposed 3N induction techniques (i.e., 4N x 2N crosses) and the commercial potential of 3N bay scallops are discussed.

RÉSUMÉ

Cogswell, A.T., E.L. Kenchington, S.E. Roach and B.W. MacDonald. 2006. Triploid bay scallops (*Argopecten irradians*): induction methodology, early gonadic development and growth. Can. Tech. Rep. Fish. Aquat. Sci. 2635: v + 48 p.

Les huîtres du Pacifique triploïdes (3N) (*Crassostrea gigas*), représentent plus de 50% de la production totale d'huître aux États Unis. Le succès de cette espèce a créé un intérêt pour la production de 3N chez d'autres espèces commerciales de mollusques, telles que les pétoncles, les palourdes et les moules. Nous rendons compte ici des tentatives d'induction de triploïdie chez le pétoncle de baie, *Argopecten irradians*. La méthode la plus communément utilisée pour l'induction 3N, nécessite l'exposition des embryons précoce aux produits chimiques (par exemple, la Cytochalasine B (CB)). La CB empêche l'élimination du deuxième corps polaire juste après la fertilisation, causant la conservation des deux paires de chromosomes femelles en plus de l'ensemble des chromosomes masculins. Ceci est réalisé en perturbant la polymérisation de l'actine. Quatre concentrations de CB ont été testées afin d'évaluer leur capacité a produire des larves 3N. Les concentrations de 0,1, 0,5 et 0,75 mg/l de CB, ont permis de créer ~25%, ~40% et ~60% de larves 3N stage D au jour 4 respectivement. Au cours de la production commerciale, une induction de 80% est considérée comme réussie. Ceci a été obtenu en utilisant une concentration de CB de 1 mg/l qui a produit 97-100% de larves 3N stage D au jour 4. Ces larves (à la concentration de 1 mg/l) ont été surveillées pendant 18 mois du stade juvénile à l'adulte, et les taux d'induction n'ont pas sensiblement diminué. Il n'y avait aucune différence de croissance larvaire entre les 3N et les 2N contrôles entre 6 et 13 jours après la ponte. Les résultats de croissance pour les pétoncles triploïdes et contrôles qui étaient transplantées dans un site d'aquiculture n'ont montré aucune différence significative dans les dimensions de la coquille après 3 mois. Cependant, le groupe 3N a montré une augmentation significative d'index de muscle de 8% ((poids sec muscle/poids sec tissu totale)*100) compensé par un déclin inversement proportionnel d'index de gonade de 6% par rapport aux diploïdes (2N). Les 3N peuvent donc partitionner leurs réserves d'énergie de façon unique. Le développement reproductif a été sévèrement compromis chez les progénitures 3N alors qu'à la même période les 2N étaient à leur pic de reproduction. Les animaux 3N sont restés hermaphrodites, alors que l'examen histologique des gonades a montré que la dimension des oeufs était sensiblement accrue (~1,2x) et que le développement du sperme semble s'arrêter au stade de spermatocyte primaire. De plus, la fécondité des oeufs était très réduite et les petits follicules semblaient être remplis d'haemocytes phagocytaires, ce qui suggère une faiblesse des follicules et la réabsorption continuelle des ovocytes. L'efficacité des techniques d'induction 3N courantes et à venir (c.-à-d., croisement 4N x 2N) et le potentiel commercial des pétoncles de baie 3N sont discutés.

INTRODUCTION

The bay scallop, *Argopecten irradians,* has a natural range that includes the Gulf of Mexico from Texas to Florida and along the entire east coast of the United States as far north as Maine. It is a hermaphroditic species commonly found in subtidal eel grass beds (*Zostera marina*) of the shallow warm waters (<10 m, 24-26°C) of protected bays and estuaries (Robert 1978; Gosling 2003). Historically there has been an active but variable commercial bay scallop fishery in the United States and more recently a boom in Chinese aquaculture with animals imported from Nova Scotia, Canada. Due to the traditionally high market value and suitability for aquaculture, the refinement of bay scallop culture techniques has been ongoing for quite some time (Castagna 1975); however, recently in the United States there has been renewed interest in the hatchery production of bay scallops for the purposes of a recreational fishery because of sharp population declines in wild stocks due to coastal eutrophication, over fishing and loss of habitat (Leavitt and Karney 2001).

Robert (1978) conducted an initial biological assessment of the suitability of bay scallops in Maritime waters. The conclusion of this assessment was that bay scallops were not likely to be successful in Maritime waters because of their reduced cold tolerance, their inability to cope in low salinity environments and the cool truncated Maritime summers. Nonetheless, despite their ease of culture and quick growth to market size, some believed that this species would act to complement other local commercial shellfisheries. In 1979, 60 bay scallops were successfully introduced from the National Marine Fisheries Service Laboratory, Milford, Conn., U.S.A. to the Ellerslie Fisheries Research Station, Ellerslie, P.E.I., Canada (Townshend and Worms 1983). In 1983, after 3 years and 4 generations in quarantine and a transitional hatchery, juveniles were placed in sites around P.E.I. for grow-out trials (Townshend and Worms 1983). It was not until 1984/85 that a small sample of F7/F8 generation broodstock was transferred from Ellerslie to the Pleasant Point Provincial Hatchery, Ostrea Lake, N.S. (Brian Muise, pers. comm., 2005). In 1987, animals from the Pleasant Point Provincial Hatchery were used in a series of contained preliminary site selection studies at points along the southern and Northumberland Strait portions of Nova Scotia (Mallet and Carver 1987a; 1987b). Most likely due to the limited genetic pool and high inbreeding of broodstock used in the quarantine phase of the Ellerslie introductions (i.e., only 8 animals remained from F1 to produce the F2 generation), the resulting transfers to N.S. displayed very poor over wintering qualities. For this reason, in 1990 fresh stock of unknown origin was distributed to at least two locations in N.S. (Ship Harbour and Little Harbour) and these scallops were cultivated in limited commercial quantities in 1991 (Scarratt 1992). In 1996, poor larval survival at the Ellerslie hatchery prompted two Nova Scotia bay scallop growers to transfer some broodstock to the Department of Fisheries and Oceans, in Halifax. At the time, there was concern that poor hatchery survival in P.E.I. was in part due again to the limited genetic pool of initially imported broodstock. Seed was then produced from the brood brought to the Halifax Laboratory and redistributed amongst Nova Scotia growers (Barry MacDonald, pers. comm., 2006). Currently in P.E.I., small pockets of naturalized populations have been established in some areas from the initial stock introduced in 1979 from Milford; however, the naturalized populations now present

in Little Harbour, N.S. (the grow-out site for this study) originated from the second introduction in 1990 (Paul Budreski, pers. comm., 2005). Based on the failure of previous introductions it was thought that the second batch of introductions would not survive the winter. Husbandry efforts in Little Harbour to maximize over wintering potential by group spawning winter survivors, collecting spring juveniles and retaining in cages to overcome peak predation, high winter tidal exchange rates, warm summer temperatures (>24°C) and plentiful habitat, made scallops in this harbour the exception (Paul Budreski, pers. comm., 2006).

As it turned out the first attempts at rearing bay scallops in Canada were severely hampered by four main factors: limited suitable habitat, a shortened growing season, low over wintering survival, a reportedly poor shelf life and a small genetic pool (Barry MacDonald, pers. comm., 2006). One commercial Nova Scotia grower (Lunenburg Shellfish Ltd.), while still dependant on hatcheries for over wintering and seed production, has found summer grow-out sites with suitable growing conditions and summer water temperatures reaching ~26°C and above (Andre Mallet, pers. comm., 2005). Nonetheless, despite the presence of suitable habitat resulting in exceptional summer growth rates (0.6 mm/day), the small initial spring spat size (< 3mm) and truncated summers of Atlantic Canada have thus far been prohibitive enough that scallops tend not to reach the optimal market size of >55 mm shell height in one growing season (Mallet and Carver 1987a). A majority of first year bay scallops will die shortly after their first spawning and that is why this market threshold is so important to reach within the first year of growth (Robert 1978; Mallet and Carver 1987a; 1987b).

It has been suggested that certain biotechnological techniques that have been shown to enhance growth in some bivalves (e.g., triploidy induction) may be the boost needed to reach or exceed optimal market size in the allotted time frame of an Atlantic Canadian summer. Even if the attempts of local growers to maximize growth by altering husbandry techniques are successful, triploid (3N) bay scallops could potentially reach a larger market size faster and provide local growers with an edge over foreign competition.

TRIPLOIDS: INCEPTION AND APPLICABILITY

In 1981, Stanley *et al.* published the first paper describing 3N shellfish production for the American oyster (*Crassostrea virginica*). Initial interest in 3N shellfish likely stemmed from observed growth increases for some scallop, oyster and clam species (i.e., *Argopecten irradians/ventricosus*, *C. virginica* and *Mulinia lateralis*) (Stanley *et al.* 1984; Tabarini 1984; Guo and Allen 1994a; Ruiz-Verdugo *et al.* 2000); however, 3N sterility and the resultant meat quality improvement during peak spawning season for some species (*C. gigas, Mytilus galloprovincialis,* and *M. edulis*) have been crucial factors spurring further research into the viability of commercial 3N production (Allen 1987; Davis 1997; Nell 2002; Brake *et al.* 2004).

Gonadic development in diploid (2N) bay scallops is controlled mostly by two contributing factors, food availability and water temperature (Sastry 1968). For this

reason, bay scallop spawning cycles vary considerably over their large latitudinal range (Sastry 1970; Barber and Blake 1983). Despite latitudinal variation, when regional waters reach optimal temperatures (~24°C) and food concentration/composition, somatic tissue (e.g., digestive gland and adductor muscle) energy reserves in the form of mainly glycogen and protein are incrementally reallocated to gonadic growth (Sastry 1970; Barber and Blake 1981; Epp *et al.* 1988). This energetic displacement tends to result in stunted shell growth, a dramatic decline in somatic tissue indices and a conversely dramatic incline in gonadic indices during peak spawning period (Sastry 1970; Barber and Blake 1981; Epp *et al.* 1988).

Unlike 2N shellfish, 3N's are usually gametogenically suppressed due to their inability to properly synapse during early prophase of meiosis I (Beaumont and Fairbrother 1991; Maldonado-Amparo and Ibarra 2002a; 2002b). For this reason some species of 3N bivalves show continued somatic growth during times of peak 2N gonadic development, potentially resulting in animals that reach market size ahead of their 2N counterparts (Stanley *et al.* 1984; Tabarini 1984; Guo and Allen 1994a; Ruiz-Verdugo *et al.* 2000; Brake *et al.* 2004). While stunted gonadic development and improved somatic growth has been noted for 3N *A. irradians* during peak spawning periods (Tabarini 1984), in-depth histological and gamete ultrastructural studies have yet been conducted. Nonetheless, a great deal is known about 3N gonadic development for the Catarina scallop (*A. ventricosus=circularis*), a hermaphroditic species of the same genus. Similar to mussels (*Mytilus galloprovincialis* and *M. edulis*), gametogenesis in the 3N Catarina scallop seems to progress only to the early prophase stage of meiosis I that corresponds with a "meiotic checkpoint" called the "recombination or pachytene checkpoint" (Kiyomoto *et al.* 1996; Roeder and Bailis 2000; Maldonado-Amparo and Ibarra 2002b). Roeder and Bailis (2000) showed that this checkpoint could halt meiosis I at the pachytene stage if sister chromatids did not properly synapse or recombine. The checkpoint appears to be a "control mechanism" that minimizes chromosome mis-segregation that would inexorably lead to aneuploid gametes (Roeder and Bailis 2000). This checkpoint is likely the cause of incomplete sperm and egg maturation of 3N Catarina scallops (Maldonado-Amparo and Ibarra 2002a). In addition, it has also been noted that the typically male acini of the hermaphroditic Catarina scallop convert to oocyte development after the immature primary spermatocytes have been absorbed. The reason for this conversion is speculative but could involve the inhibition of synthesis for certain endocrine signals that may be responsible for regulating the male component in this species (Gomot and Griffond 1993; Maldonado-Amparo and Ibarra 2002b). Light microscopy observations of early gonadic histological sections for bay scallops grown in the field will provide more insight into early gametogenesis for both 2N's and 3N's.

According to Beaumont and Fairbrother (1991) the resulting sterility caused by 3N induction is commercially important for 3 main reasons: first, energy in the form of glycogen usually reserved for gonadal development can be redirected to somatic tissue leading to elevated somatic glycogen content and increased somatic growth. Second, poor condition of some species (*M. edulis* and *C. gigas)* pre- or post-spawn will negatively impact appearance, taste and/or meat yield, thus reducing their marketability (Allen and Downing 1991; Brake *et al.* 2002). Triploid shellfish mitigate this problem due to retarded gonadal development and the subsequent somatic reallocation of glycogen reserves which in some cases leads to improved taste and year-round marketability (Allen

and Downing 1991). Thirdly, when intentionally or incidentally introduced into established local populations, sterile 3N organisms would reduce introgression of foreign genes (i.e. interspecific or interspecies hybridization).

TRIPLOID INDUCTION TECHNIQUES

Since 1981, 3N production methods in many species of oysters, clams, scallops and mussels have been described and refined (Allen 1987; Beaumont and Fairbrother 1991; Desrosiers *et al.* 1993; Scarpa *et al.* 1994; Davis 1997; Jackson *et al.* 2003). The most commonly employed 3N shellfish induction procedure involves retaining the second polar body of the early embryo via chemical or physical treatment. Techniques employed have included: electrofusion, cold/heat shock, caffeine, cytochalasin B (CB), and nearly any combination of the aforementioned examples (Yamamoto and Sugawara 1988; Cadoret 1992; Scarpa *et al.* 1994; Toro and Sastre 1995; Davis 1997). Surier and Karney (2004) have employed 6-Dimethylaminopurine (6-DMAP), a far less toxic chemical than CB, for successfully inducing 3N bay scallops at a rate of 77-100%. Data from trials examining their growth in the field should soon be available. Nonetheless, until recently the most successful and commercially viable method of 3N production involved treating early embryos with CB, a carcinogenic, cell permeable fungal toxin (Forscher and Smith 1988; Allen *et al.* 1989). CB exposure, in concentrations typically ranging from 0.25 mg/l to 1 mg/l, inhibits actin polymerisation and halts microtubule formation thus retaining the second polar body of the developing embryo resulting in individuals with 2 maternal and 1 paternal chromosome complement (Beaumont and Fairbrother 1991; Barber *et al.* 1992; Scarpa *et al.* 1994; Nell *et al.* 1995; Ruiz-Verdugo *et al.* 2000; Yang *et al.* 2000a; Jackson *et al.* 2003). Occasionally however, the retention of this extra chromosome set is incomplete resulting in aneuploid individuals which rarely persist through D stage metamorphosis (Guo *et al.* 1989). Although 3N induction success utilizing CB has been highly variable between species (Beaumont and Fairbrother 1991), 3N's are produced in enough quantity that this method is commercially viable. In fact, nearly 30% of all Pacific oysters farmed on the West Coast of North America in 1999/2000 were 3N's produced by this method (Nell 2002). Tabarini (1984) developed methodology to successfully produce high percentages (94%) of 3N bay scallops by exposing fertilized eggs to 0.1 mg/l CB for 20 minutes, 10 minutes post-fertilization. Despite the relative ease of 3N induction and increased somatic growth seen by Tabarini (1984), little is known about 3N bay scallops and they have only been recently re-examined (Surier and Karney 2004). Given that most species of shellfish require, at the very least, 0.25 mg/l of CB to induce optimal levels of triploidy, there is some question as to the repeatability of Tabarini's results. Experiments with the Catarina scallop used two concentrations of CB (0.1, and 0.5 mg/l) to induce triploidy, and while these concentrations were well within and above the optimal concentration suggested by Tabarini (1984), the 0.1 mg/l concentration only produced 8% 3Ns and the 0.5 mg/l treatment produced only 58% (Ruiz-Verdugo *et al.* 2000). It is in light of these observations, that this study will re-evaluate 3N induction methodology for bay scallops.

Currently, the most reliable method of 3N production involves crossing tetraploid (4N) male broodstock with 2N females to produce 100% 3N offspring (Eudeline *et al.* 2000). Technology developed to successfully produce commercial quantities of 4N Pacific and Atlantic oysters (*C. gigas and C. virginica*) is proprietary/patented and owned by 4C's Breeding Technologies, Inc (Wildwood, New Jersey). The most successful techniques employed to produce 4N broodstock, at least in Pacific oysters, requires inhibiting the first polar body extrusion of eggs from 3N females fertilized with sperm from 2N's by treating them with 0.5 mg/L CB added ~10 minutes after fertilization (Guo and Allen 1994b; Eudeline and Allen 2000). This method, while applicable for Pacific oysters, is species dependent and will be useless when applied to species, that when induced for triploidy, produce only sperm or severely retarded oocytes (Kiyomoto *et al.* 1996; Ruiz-Verdugo *et al.* 2000). Whether this method is applicable to 3N bay scallops is entirely dependent on their ability to spawn viable eggs at any point during gametogenesis. Another method of 4N production proposed by Scarpa *et al.* (1993) involved exposing fertilized eggs from 2N *M. edulis* females to 1 mg/l CB from 7 until 35 minutes post-fertilization, blocking both the first and second polar body division, which resulted in 24.1% - 2N, 58.7% - 3N and 17.2% - 4N spat. This is a method that may have some potential with bay scallops, especially given their relatively quick first and second polar body release time (<20 minutes). A short meiotic stage could reduce CB exposure time potentially resulting in higher survival than that seen in mussels which would require a more extensive exposure time. Due to the highly toxic nature of CB, it is likely that in the years to come chemical and procedural alternatives to CB will be necessary for commercial production. The production of 3N's through 4N x 2N matings may be the next step in producing large numbers of 3N bay scallops without the necessity of handling harmful chemicals. While exposure to CB in concentrations used to induce triploidy are potentially harmful to human health, studies have yet to be designed to assess the persistence of CB in market-sized shellfish.

Another 3N induction technique that has shown promise in recent years involves exposing fertilized eggs to 6-DMAP. This chemical is much less toxic than CB and has been shown in some species to induce nearly 100% triploidy (Davis 1997). Davis (1997) has modified chemical induction techniques developed by Desrosiers (1993) which involves treating fertilized *M. galloprovincialis* eggs with a combination of heat shock (5-10 °C above ambient) and 6-DMAP (30-300 μM) during the period corresponding with second polar body release. According to Davis, this results in nearly 100% 3N larvae and juveniles. This method can now be used to treat commercial amounts of *M. galloprovincialis* eggs (100-300 million). Recently, Surier and Karney (2004) used similar methodology, employing the use of 6-DMAP (400 μM) during early development (15-25 minutes post-fertilization for 11 minutes) to produce between 77-100% 3Ns.

Given the elevated somatic growth seen in 3Ns of the *Argopecten* genus (*irradians and ventricosus*) and the ease of which these 3Ns can be produced by various methods, there is a need in assessing their field performance in relation to their 2N counterparts (Tabarini 1984; Ruiz-Verdugo *et al.* 2000; Surier and Karney 2004).

MATERIALS AND METHODS

CYTOCHALASIN B TRIPLOID INDUCTION

Spawn I and ploidy assessment

On March 11th, 2004 15 bay scallops collected from Aqua Delight Seafoods Ltd. (Fig. 2 - #1224) lease site in Little Harbour, N.S. on December 23rd, 2003, were spawned for the first of 2 triploidy induction trials. Each bay scallop was placed into individual 250 ml plastic containers filled with 25°C 1µm filtered UV treated seawater (treated seawater) to induce spawning. Approximately 13/15 bay scallops spawned male first and sperm was pooled while scallops were rinsed with salt water and placed back into freshly exchanged filtered seawater. Once eggs were seen being expelled into the containers, scallops were removed, cleaned with freshwater and placed into new containers of treated seawater. Despite careful rinsing, upon the completion of spawning microscopic examination revealed that each egg batch had experienced varying degrees of self-fertilization.

Two egg batches displaying the lowest levels of self-fertilization (~1 sperm/5 eggs) were used in the preliminary test of Tabarini's (1984) 3N induction methodology. Eggs from the first individual (2.2×10^6) were placed into a 1L beaker containing 200 mls of 25°C treated seawater and fertilized with sperm at a concentration of 10-15 sperm/egg. The beaker was then filled with 800 ml of treated seawater. After microscopically observing ~50% first polar body release (10 minutes post-fertilization), 100 µl of CB solution (1 mg CB/ml Dimethyl Sulfoxide (DMSO)) diluted into 200 mls of treated seawater was added to the embryos for a final CB concentration of 0.1 mg/l. During the 15 minute treatment, embryos were stirred every 1-2 minutes to maintain a homogeneous exposure environment. Post-treatment, embryos were thoroughly rinsed on 20µm Nitex mesh with treated seawater to remove the bulk of the CB. Embryos were then re-suspended in a 1 L 0.02% DMSO solution for 15 minutes and rinsed on Nitex mesh to remove any persisting traces of CB. Upon completion of rinsing, embryos were placed into a 250 L tank containing 20°C 1µm filtered UV treated seawater and monitored for development and early survival. This procedure was repeated with ~2.0×10^6 eggs from a second individual using a CB concentration of 0.5 mg/l CB. Two controls were created from the remaining eggs with the first control consisting of ~3.1×10^6 severely self-fertilized eggs and the second control comprised of pooled groups of partially self-fertilized eggs (~7.7×10^6) that had been exposed to pooled sperm shortly after they had been microscopically assessed as self-fertilized. Controls were placed into two separate 250 L tanks filled with 20°C 1µm filtered UV treated seawater for larval rearing. Scallops in each bucket or tank were fed daily with consistent concentrations of a combination of algae (*Chaetoceros muelleri*, *Isochrysis galbana*, *Tetraselmis sp.*) to satiation. The quantity and composition of the algae depended primarily on larval clearing rates and algae availability.

On day 4 (March 15th, 2004), control and treatment tanks were drained onto 40μm mesh and 3 larval samples, enough to form a small pellet in each 1.7 ml Eppendorf tube (~1000 larvae/tube), were collected for flow cytometric ploidy assessment. Flow cytometry protocols refined by Jackson *et al.* (2003) were employed to determine 3N induction rates for both the 0.1 mg/l and 0.5 mg/l CB treatments (Appendix 1). Utilizing similar methodology, set larvae were taken from the 0.5 mg/l treatment at day 26 post-spawn to determine the ploidy induction level. Data was initially collected and analysed using Cell Quest Pro software (Becton Dickson and Company, San Jose, California) prior to further scrutiny via ModFit LT Software (Verity Software House, Inc.). ModFit LT has the capacity to normalize and differentiate 2N and 3N peaks and to more accurately describe 3N induction success.

Spawn I larval rearing

D-larvae were visible in only the control and 0.1 mg/l group 24 hours after fertilization; however, due to poor survival (25%) and D-larvae formation (<60%) scallops from both control tanks were merged into one 250 L tank at a final density of ~10 larvae/ml. It was also clear that early development to D-larvae was delayed in the 0.5 mg/l treatment and many deformed swimming trochophores were visible. Day 4 post-spawn, ploidy confirmation via flow cytometry revealed little or no 3N induction in the 0.1 mg/l treatment; thus, this treatment was discarded. Survival for the 0.5 mg/l treatment, while visibly poor at both day 1 and 4 post spawn, was not recorded during larval grow-out because poor 3N induction rates observed did not warrant continual monitoring of their progress.

Spawn II and ploidy assessment

Due to the relatively poor 3N induction success observed for both treatments from spawn I, a second spawn was conducted using 14 bay scallops on April 15th, 2004. During spawn I it was not possible to collect eggs from one individual that were entirely devoid of their own sperm. To enhance the probability of eggs being fertilized with sperm from another male, 3 groups of bay scallops (5, 5 and 4) were induced to spawn in 10L buckets filled with 25°C 1 μm filtered UV treated seawater. Because males spawned first in all buckets, there was pooled sperm available for subsequently spawning females. Approximately 9 million embryos were collected from one bucket shortly after females began spawning. The 0.75 mg/l treatment involved ~2.5 x 10^6 embryos treated with CB at 50% first polar body release. Upon completion of a series of mixes and rinses, as described in spawn I, embryos were placed into a 250 L tank. The 1 mg/l treatment was performed in duplicate. At 50% first polar body release embryos were treated with 1 mg/l for 15 minutes. The first 1 mg/l CB treatment involved ~1.1 x 10^6 embryos that upon rinsing were evenly split into 2 – 20 L buckets. The second 1 mg/l treatment was

performed upon ~2.5 x 10^6 embryos that, after rinsing, were placed into a 250 L tank filled with 20°C treated seawater. In addition, ~2.5 x 10^6 untreated embryos from a 10L spawning bucket were placed into a 250 L tank. All treatment and control larvae were reared in 20°C 1µm filtered UV treated seawater and were fed with consistent concentrations of algae as described for spawn I.

Day 4 post-spawn, flow cytometric procedures as described for Spawn I were performed on larvae from all treatment and control tanks (Appendix 1). Ploidy was periodically assessed on larvae of various stages and individual juveniles throughout 2004-2005 while being reared in the wet lab at the Bedford Institute of Oceanography (BIO), Dartmouth, N.S. (Appendix 2).

Spawn II larval rearing

After 24 hours post-fertilization early development was assessed for each treatment and the control. On day 4 post-spawn (April 19th, 2005) D-larvae survival was assessed in all treatment and control tanks from spawn II. Due to low survival in the 1 mg/l treatments, larvae from the 250L tank and 2 buckets were merged into one 250L tank. Severely reduced survival in the 0.75 mg/l treatment meant that the remaining animals could be reared in a 20L bucket. The 0.75 mg/l treatment was not followed beyond day 4 because flow cytometry ploidy confirmation on day 4 revealed less than optimal 3N induction rates. Early larval growth was assessed by measuring shell height for both the control and 1 mg/l treatment groups 6, 8 and 13 days post-fertilization while survival was assessed at 4 and 6 days post-fertilization for both groups. Survival was not assessed more frequently because numbers were already low in the 1 mg/l treatment group.

Spawn II spat and juvenile rearing

On May 5th, 2004 (day 20 post-fertilization) spat were seen setting in both treatment and control groups. At this time, one small pellet of larvae from each of the control and treatment groups was taken for ploidy analysis. In addition, on May 17th 2004 (day 32 post-fertilization) scallops that had metamorphosed in both the 2N and 3N group were separated from non-metamorphosed scallops. The metamorphosed scallops consisted of scallops from ~210µm to 600µm and the non-metamorphosed scallops were <210µm. Samples of the non-metamorphosed and metamorphosed scallops from the control and 1 mg/l treatment were taken for ploidy analysis. As scallops continued to set they were moved into downwellers suspended in 250L tanks for further rearing. On July 7th (day 53 post-fertilization), 6 downwellers (3 control downwellers and 3 - 1 mg/l treatment downwellers) were moved from 3 separate 250L tanks to a common 1000L tank and reared at 19°C. At this time, 2N downwellers had to be thinned out

considerably to reach a comparable biomass to that seen in the CB treatment downwellers.

Starting September 2nd, 2004, 2N and 3N bay scallops were placed into a 1200L tank containing 6 – 3 mm pearl nets each with 400-800 bay scallops, for a total of 3200 CB treated and 3300 untreated scallops (Fig 1). The total amount of control and treated scallops remaining before being placed into pearl nets were ~4700 and ~4000 consecutively. This number is not indicative of overall survival to this point because 2N's had been graded considerably to match the lower stocking densities seen in the 1 mg/l treatment group. To meet the feeding demands of growing scallops and reduce stocking density stress, scallops were thinned periodically over the next 7 months as recorded in Appendix 3.

As a requirement of Introductions and Transfers, Department of the Fisheries and Oceans (DFO) Maritimes Region it was necessary to obtain full disease profiles for samples of shellfish to be transferred from the BIO wet lab to Little Harbour, N.S. On April 18th, 30 2Ns and 30 CB treated scallops (5/pearl net) were assessed for ploidy height, length, width, whole weight, tissue weight and shell weight. Whole tissue from each scallop was excised and placed into histology micro-cassettes (Surgipath Medical Industries, Inc., Winnipeg, Manitoba) labelled for each individual using a case number, specimen number, abbreviated species ID and date sampled. Labelled cassettes were then transferred into 500 ml square plastic bottles filled with Davidson's solution (Appendix 4) for 24 hours. After 24-48 hours samples were removed to 70% ethanol and shipped to the DFO, Shellfish Health Unit, Moncton, N.B for full disease profiling (Appendix 5).

DIPLOID AND TRIPLOID FIELD TRIALS

On May 31st, 2005 CB treated and control scallops were transferred to lease site #1224 in Little Harbour, Pictou County, Nova Scotia (Fig 2). This is a protected, shallow (< 2 feet water depth at low tide), warm water (~24°C during summer peak) harbour with abundant eel grass (*Zostera marina*), all of which are conducive to successful bay scallop culture (Gosling 2003). Approximately 600 scallops/treatment had been available a month earlier, but due to a rather intense mortality event, likely attributable to overstocking under laboratory conditions, 120 CB treated scallops and 120 control scallops were deployed (Fig 3). Treated and untreated scallops were placed into separate ¼" polyethylene mesh bags at 20/bag to minimize stocking density effects on growth. The resulting 6 bags/treatment were secured by cable ties to three rebar racks covered in 2 inch polyethylene mesh (Fig 4). Rack 1 consisted of 5 bags, 3 CB treatment bags (3N1, 3N2 and 3N3) with 2 2N bags (2N1 and 2N2) placed between each CB treatment. Rack 2 was comprised of the other 3 CB treatment bags (3N4, 3N5 and 3N6) and the other 2 2N bags (2N3 and 2N4) interspersed between them. The third rack contained the remaining 2 2N bags (2N5 and 2N6).

On the day of deployment (May 31st, 2005) water temperatures were recorded and 30 scallops from each treatment were taken to BIO for analysis. Fifteen scallops from each treatment were assessed for: shell height, length, width, whole weight, gonad wet/dry weight, adductor muscle wet/dry weight, remaining tissue (rest) wet/dry weight, shell weight and ploidy level. Wet tissue was placed on a tinfoil weigh boat and put into a drying oven at 70°C until a constant weight was achieved (~24 hours). Dry weights were used to calculate the gonadic and somatic indices of each scallop as an indicator of each tissues relative condition. Condition indices (Gonado-Somatic, Muscle and the remaining tissue (or Rest) indices) were calculated using the following formula (Gonado-Somatic Index (GSI) as an example):

$$GSI = (\text{Gonad dry weight} / \text{total tissue dry weight}) * 100$$

The remaining fifteen scallops from each treatment were assessed for: shell height, length, width, whole weight, tissue wet weight, shell weight and ploidy level. As previously described, whole tissue from each of these 15 scallops from each treatment was excised, placed into cassettes, fixed and sent to the DFO, Shellfish Health Unit, Moncton, N.B. for histological preparation. Examination of gonad development via histology provides a benchmark for comparisons with dry weight gonad indices. Slides for both treated and untreated scallops were scored for sex, level of gametic development and any obvious disruptions of normal gametogenesis.

Due to high mortality (~50%) observed in all nets on the subsequent sampling date (June 27th, 2005), scallops of similar treatment (CB treated or 2N controls) from each rack were pooled and 15 scallops from each treatment/rack measured for shell height, length and width. In an effort to maintain enough animals for histological preparation and tissue indices analysis during the late summer sampling date (August 11, 2005) scallops could not be destructively sampled during the June 27th or July 18th sampling dates. On June 27th, 2N and CB treated scallops were restocked in 3 - ¼" mesh nets each at 20/net (Fig 4). These six nets (3 2N and 3 CB treated) were placed on one rack for grow-out until the next sampling date (July 18th, 2005). On July 18th, mortality for each net was recorded and every scallop from each net was measured for shell height, length and width. On August 11th, mortality in each net was recorded and all remaining 2N and CB treated scallops were collected from the Little Harbour site in individual labelled bags for each net/treatment. Half of the scallops in each labelled bag were sampled for shell height, length, width, whole weight, tissue wet weight, shell weight and ploidy. Tissue from these scallops was sent for histological preparation as described in the previous section. The remaining scallops from each bag were measured for shell height, length, width, whole weight, gonad wet/dry weight, adductor muscle wet/dry weight, rest tissue wet/dry weight, shell weight and ploidy.

Statistical analysis of growth parameters was carried out using the JMP 5.1 statistical package (SAS Institute, Cary, NC, USA). Each parameter was tested for normality using the Shapiro-Wilk's test. Each growth parameter was log-transformed with the exception of percentage values which were arcsin-transformed. Best fit comparisons between non-transformed and transformed data, in addition to Levene's test for variance revealed which data were suitable for comparison by two-way ANOVA with treatment and time as factors. If no "treatment x time" interaction effect was evident, then the ANOVA was conducted removing the interaction term. An additional test (Chen

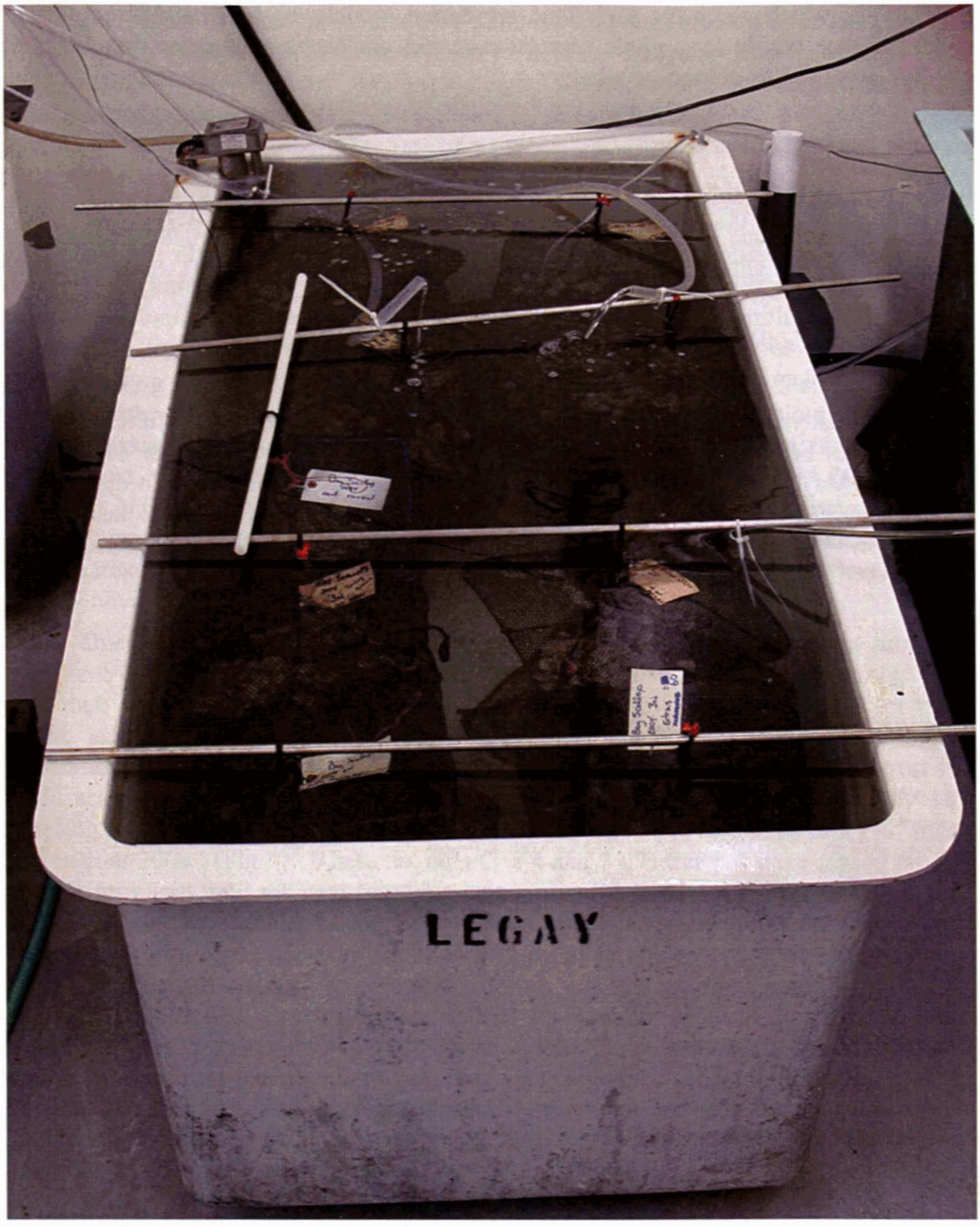


Figure 1 - 1200L holding tank for 2N and 3N bay scallop prior to field deployment.

Figure 2 - A Google Earth satellite view of Little Harbour, Nova Scotia, overlaid with lease site map (#1224) available from the Nova Scotia Agriculture and Fisheries Web Site (http://142.176.62.101/fishery).

Figure 3 - Size of 2N and 3N bay scallops one month after being deployed to the field (June 27th, 2005). Poor growth and survival was seen one month post-deployment.

RESULTS

CYTOCHALSIN B TRIPLOID INDUCTION TRIALS

Assessments of the ploidy induction rates on Day 4 after both the first (March 11th, 2004) and second spawn (April 15th, 2004) revealed an optimal 3N induction treatment of 1 mg/l CB at ~50% first polar body release for 15 minutes prior to a 15 minute 0.02% DMSO rinse. At day 4, ~96% 3N D-larvae were observed for this treatment. The DNA index (DNAI) seen at the top of each bar in Figure 5 is a measure of relative DNA fluorescence, correlating to DNA content between two distinct groups of nuclei seen as peaks in Figure 6 as measured by ModFit LT. Relative fluorescence intensity correlates with greater nuclear DNA content; thus, nuclei from the 1 mg/l treatment 3N peak mean contain, on average, 1.5 times more DNA than their corresponding 2N nuclei peak. A DNA index less than 1.5 indicates aneuploid nuclei with incomplete retention of the second polar body chromosome set after exposure to CB. Aneuploid nuclei appear in relatively large numbers (~20%) in controls at day 4 post spawn (Fig 5). As CB concentrations near optimal (1mg/l), DNA index increases towards a maximum of 1.5 and the relative proportion of aneuploids in the non-diploid peak decreases (Fig 6). Triploidy induction rates for the 1 mg/l treatment were assessed periodically while scallops were held at BIO and during 2005 field trials (Fig 7). These results show a reduction of aneuploid nuclei in 2N control larvae to ~3% in less than 2 months. May 17th (day 32 post-fertilization) ploidy analysis of metamorphosed and non-metamorphosed larvae from the CB treatment group revealed a 95% 3N induction rate regardless of the rate of larval development. The 1 mg/l treatment showed consistent induction rates over the duration of their measurements with only a 5% decrease from day 4 values at over a year and a half later (August 11th, 2005).

Survival was reduced to the D-larvae stage in all CB treatments as compared to the control. Due to the poor induction rates seen in the 0.1 mg/l and 0.5 mg/l treatments from the first spawn, survival was only noted for the 0.75 mg/l treatment to day 4 and to day 6 for the control and 1 mg/l treatment (Fig 8). Figure 8 shows an ~81% mortality rate 4 days post-spawn for the control as compared to ~94% and 97% for the 1 mg/l and 0.75 mg/l treatments respectively. While 1 mg/l treatment mortality was ~12.5% greater than the corresponding 2N controls over the first four days, both groups experienced another ~1.5% decline by day 6 post-spawn. This suggests, that while a treatment effect on early larval development and survival is evident, exposure to CB does not noticeably effect survival past D-stage. In addition, despite poorer CB treatment survival to day 4 post-spawn, no significant difference in larval shell height was noted between 4 and 13 days post-spawn (Fig 9).

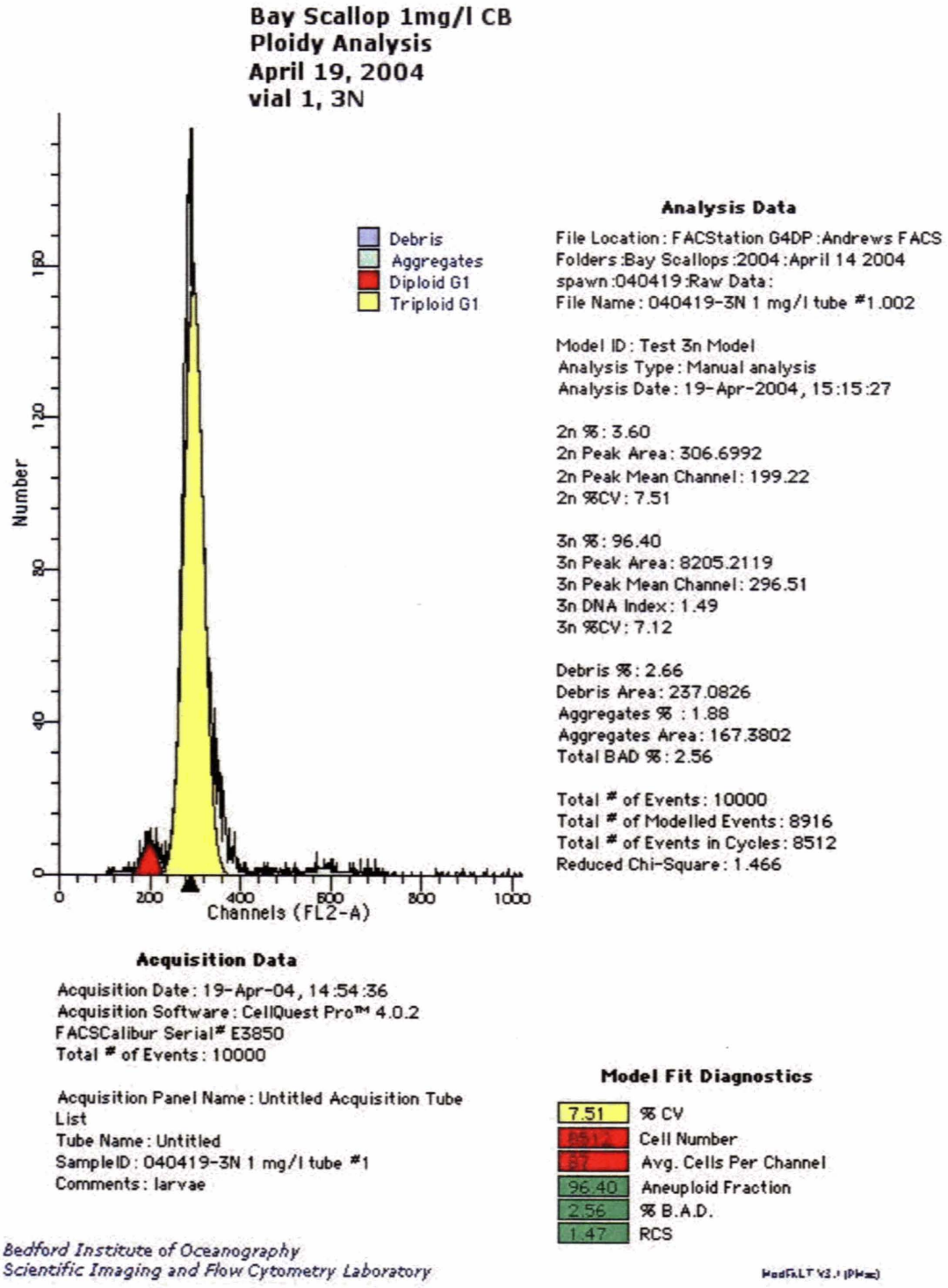


Figure 6 - Modfit LT analysis of D-larvae nuclei from vial 1 day 4 post-spawn. The fluorescence intensity of the yellow peak (3N) is 1.49 times greater than the red peak (2N).

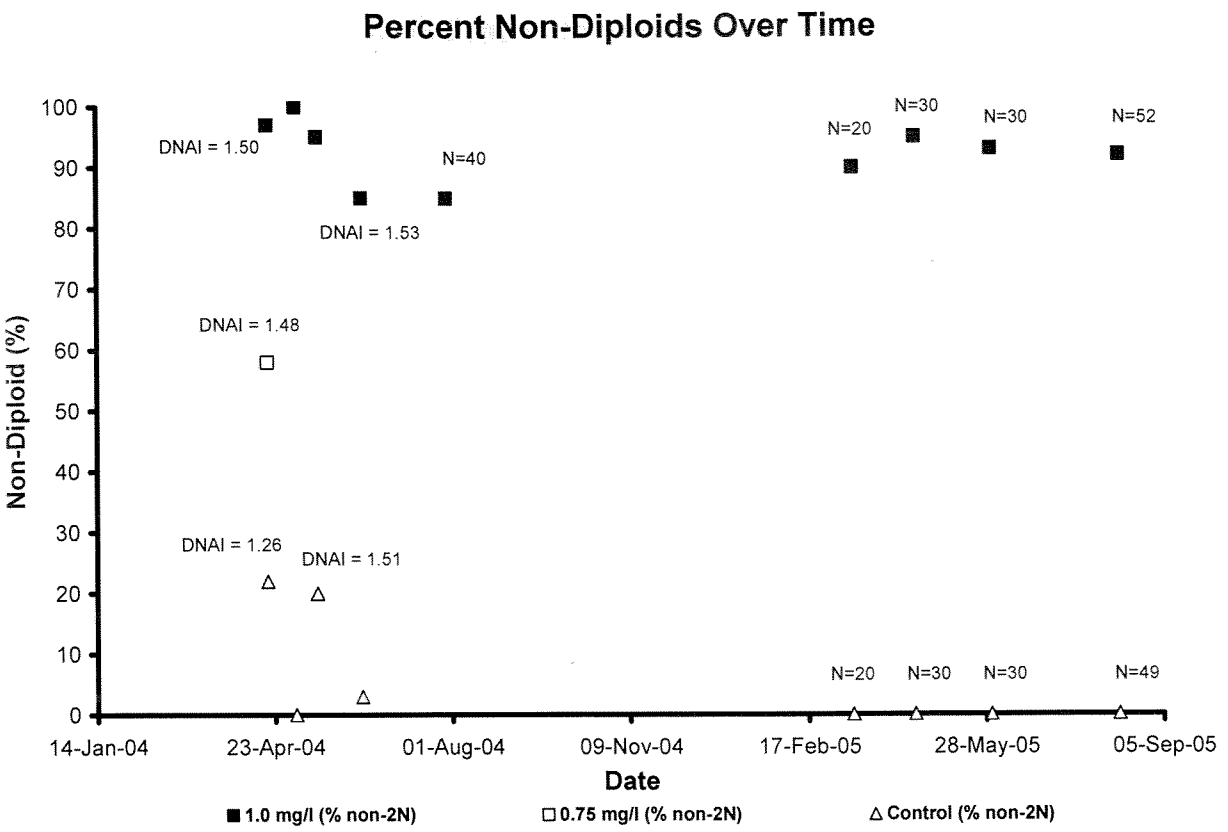


Figure 7 - Triploidy induction rate (% non-diploid) over the duration of experimental observations.

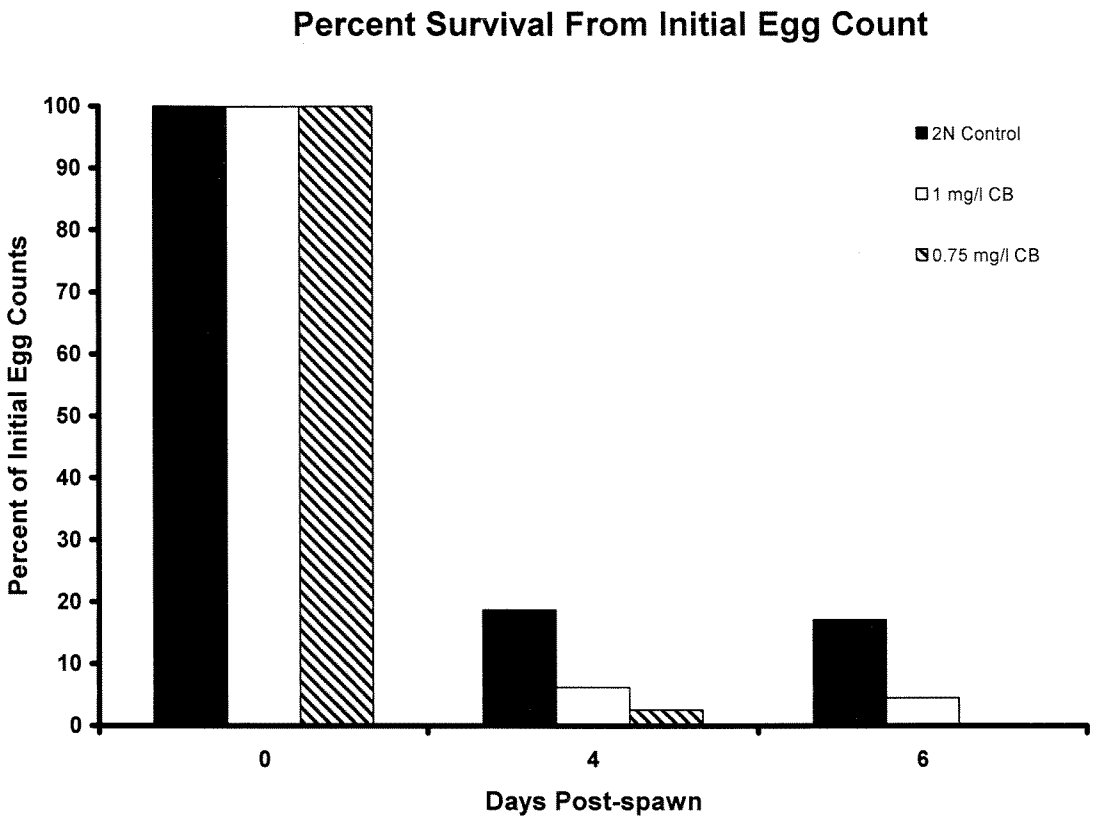


Figure 8 - Early larval survival after exposure to two concentrations of CB as compared to the 2N control.

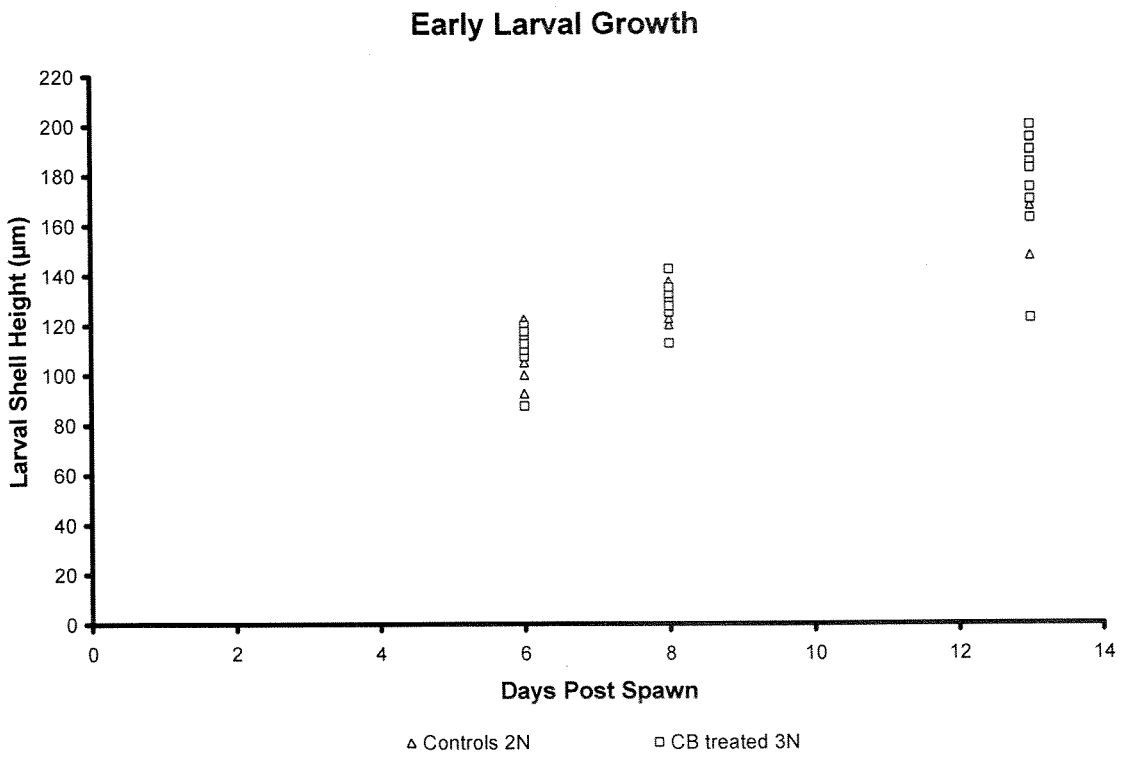


Figure 9 - Early growth, as measured by shell height of 2N and 3N larval bay scallops.

DIPLOID AND TRIPLOID FIELD TRIALS

Growth and Survival

Prior to being placed in the field, samples sent to the DFO Shellfish Health Unit for disease profiling showed no significant difference in any growth parameters; however, the profiling did reveal 5 2Ns and 3 3Ns infected with *Perkinsus karlssoni*, a non-lethal lesion-forming parasite (McGladdery *et al.* 1991) that has been identified previously in the scallops taken from lease site #1224 (Mary Stephenson, pers. comm., 2005).

On the June 27th sampling date, mean mortality rates for all nets (N=6/ploidy) were high for both 2Ns (30±15.5%) and 3Ns (46.3±12%). Only 1 mortality (3N group) was seen in either ploidy for the July 18th sampling date and only 6 3N and 5 2N mortalities were observed on the August 11th sampling date.

While ANOVAs comparing shell parameters indicated that 2Ns were significantly ($p<0.05$) larger in terms of shell height and length (Figs 10 and 11) over the entire duration of the field observations (May 31st – August 11th), further tests revealed that due to the large variability for growth parameters at each sampling date a single regression line was significantly more effective ($p<0.01$) at describing shell height, length and width (Fig 12) data for both ploidies than 2 separate regression lines (Table 1).

At deployment on May 31st there were no significant differences for any body indices, between 2N and 3N bay scallops (Table 2). On August 11th (72 days post-deployment), the means for both GSI and muscle indices (MI) were significantly different (Bonferroni correction, $\alpha = 0.008$) between the untreated 2Ns and the CB treated 3Ns (Table 2). Mean GSI for 2Ns was ~6% greater than 3N GSI. Inversely, mean 3N MI was ~8% greater than 2N MI. Rest indices did not significantly differ between 2Ns and 3Ns over the duration of the field observations. Elevated 2N GSI on August 11th was further supported by histological observations which showed enhanced gametic development as compared to their 3N counterparts (Figure 13). Nonetheless both 2Ns and 3Ns showed a significant increase in the proportion of total tissue dry weight attributable to the gonad between May 31st and August 11th (Table 3). Conversely, over the same time period 2Ns showed a significant decline in MI and RI, while 3Ns showed no significant decline in MI and a marginally significant decline in RI. This suggests that despite the significant increase in GSI for 3Ns between May and August, reserves for gonad development are not drawn from adductor muscle but from the rest tissue and filtered algae. The end result is a proportionately larger muscle size for 3N bay scallop.

On August 11th, 2005 visual examination of 2N bay scallops revealed hermaphroditic white (sperm) and orange (eggs) stage IV gonads with a thin black membrane still partially surrounding the gonad (Barber and Blake 1983). Triploid gonads during the same sampling period showed no colour and were entirely encompassed by the black membrane typical of stage III 2N gonads (Barber and Blake 1983). This suggested that 3N gonad development was retarded compared to that of 2N development during the same sampling period. This was evident in two ways: gonadic indices for 3Ns were significantly less than 2Ns for the August sampling period; and assessment of the 3N gonad histological preparations from the August sampling date,

while revealing both sperm and egg, showed retarded sperm development and significantly larger eggs ($p<0.05$, (3N (57.1±7.6 μm) and 2N (47.±5.7 μm)) which were observed at only a fraction per follicle as compared to mature 2Ns (Fig 13). Triploid egg follicles tended to be inundated with haemocytes, and sperm within male follicles did not develop beyond primary spermatocytes and appeared to be stalled at the pachytene checkpoint.

No images of whole gonad condition were taken during the August 2005 sampling period, for this reason 9 2N and 15 confirmed 3N bay scallops produced by an independent study by Carver and Mallet, 2005 (pers. Comm.), using the optimal CB induction methods refined by BIO researchers (1 mg/l), were conditioned in 14 mm pearl nets placed into an insulated 250 L tank with 18°C sea water flowing at a rate of 1.5L/min. Scallops were fed to satiation with available cultured phytoplankton species over a 7 week duration starting in March of 2006. Bay scallops were sacrificed in May of 2006 and four representative 2Ns and 3Ns were photographed to provide a comparison of gonad appearance. While not at peak maturity, both 2N and 3N gonads showed egg (orange/red) and sperm (white/light grey) development (Fig 14). Results from August 2005 and May 2006, while not definitive, suggest that while 2Ns and 3Ns can both reach a state where colour is visible in the maturing gonad; 3Ns reach this stage (Stage IV) later than their 2N counterparts and it is unlikely that 3Ns would ever reach a visible state of full maturity comparable to that of 2Ns.

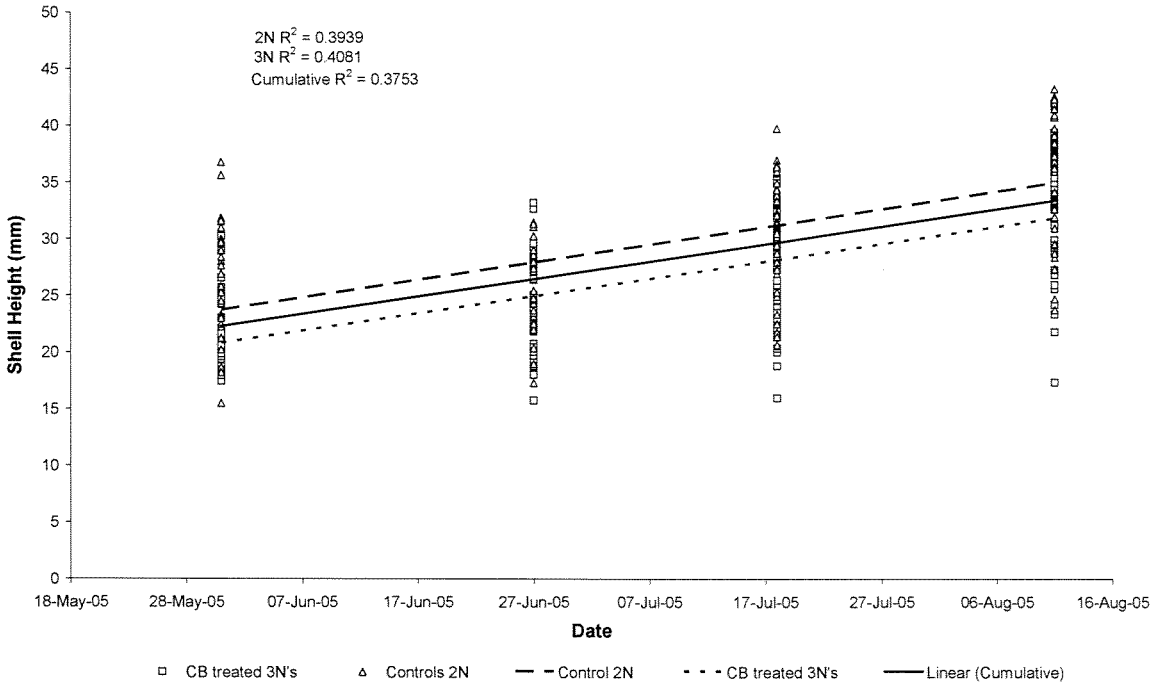


Figure 10 – Shell height for 2N and 3N bay scallops from May 31st to August 11th 2005. The black regression line represents a cumulative model for pooled 2N and 3N shell height information for each date (Table 1).

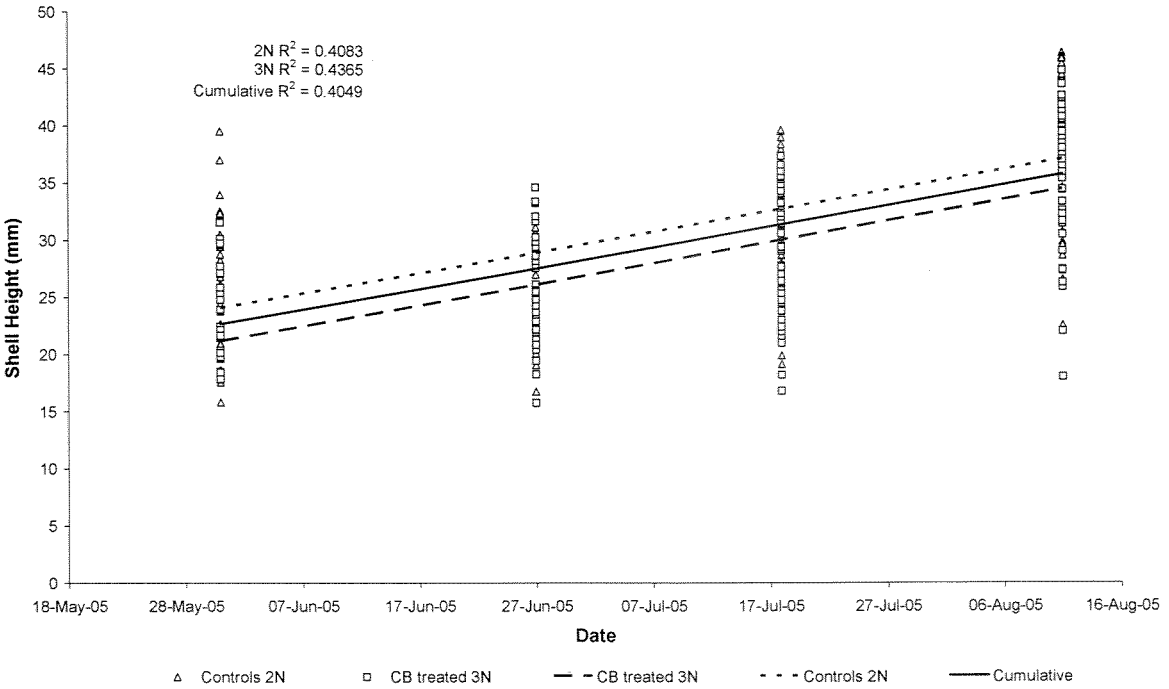


Figure 11 – Shell Length for 2N and 3N bay scallops from May 31st to August 11th 2005. The black regression line represents a cumulative model for pooled 2N and 3N shell length information for each date (Table 1).

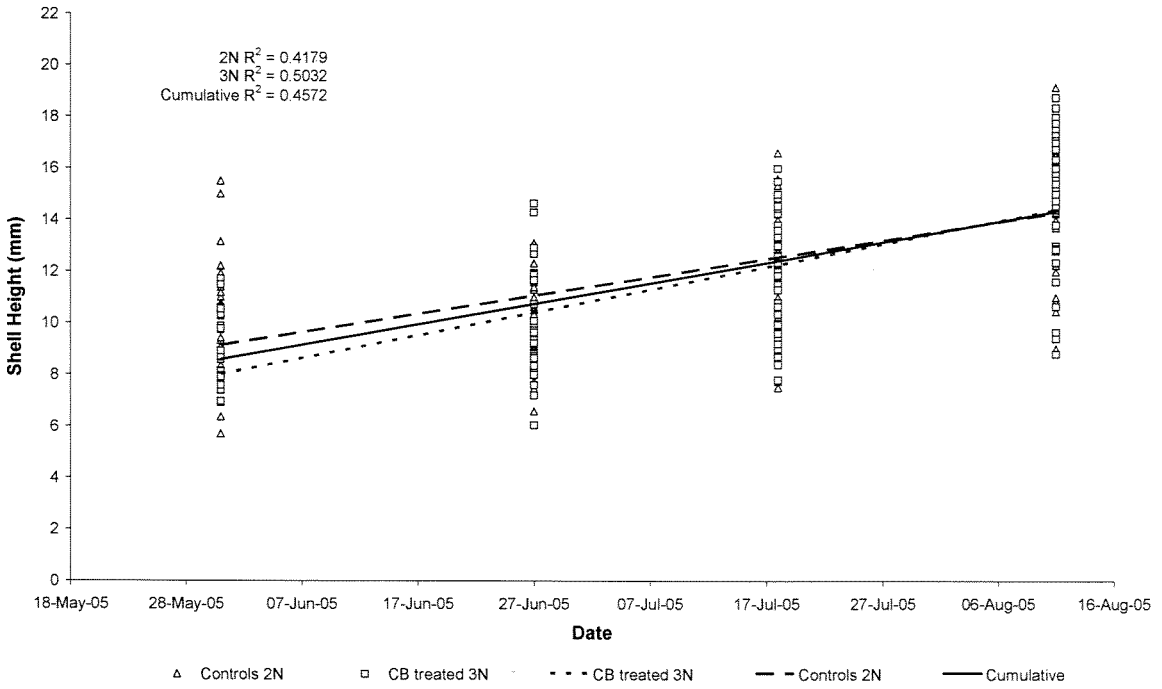


Figure 12 – Shell width for 2N and 3N bay scallops from May 31st to August 11th 2005. The black regression line represents a cumulative model for pooled 2N and 3N shell width information for each date (Table 1).

Table 1 - Ploidy regression comparisons for shell growth parameters.

Growth Parameter	RSS_p	DF	RSS_s	DF	F	$P > F_{.05}$
Shell Height	8193.94	331	7423.18	329	~ 3.00	< 0.01
Shell Length	9988.66	331	9363.38	329	~ 3.00	< 0.01
Shell Width	1561.79	331	1533.94	329	~ 3.00	< 0.01

Table 2 - Results from t-tests conducted on both May 31st and August 11th comparing the 2N and 3N means of each body indices.

Tissue Indices		May 31, 2005 Mean (SE)	P	August 11, 2005 Mean (SE)	P
Gonado-Somatic Index	2N	0.041 (.004)	0.925	0.185 (.012)	0.001**
	3N	0.041 (.005)		0.128 (.011)	
Muscle Index	2N	0.270 (.220)	0.123	0.217 (.012)	0.0001**
	3N	0.320 (.230)		0.296 (.011)	
Rest Index	2N	0.689 (.024)	0.162	0.599 (.015)	0.267
	3N	0.639 (.025)		0.576 (.014)	

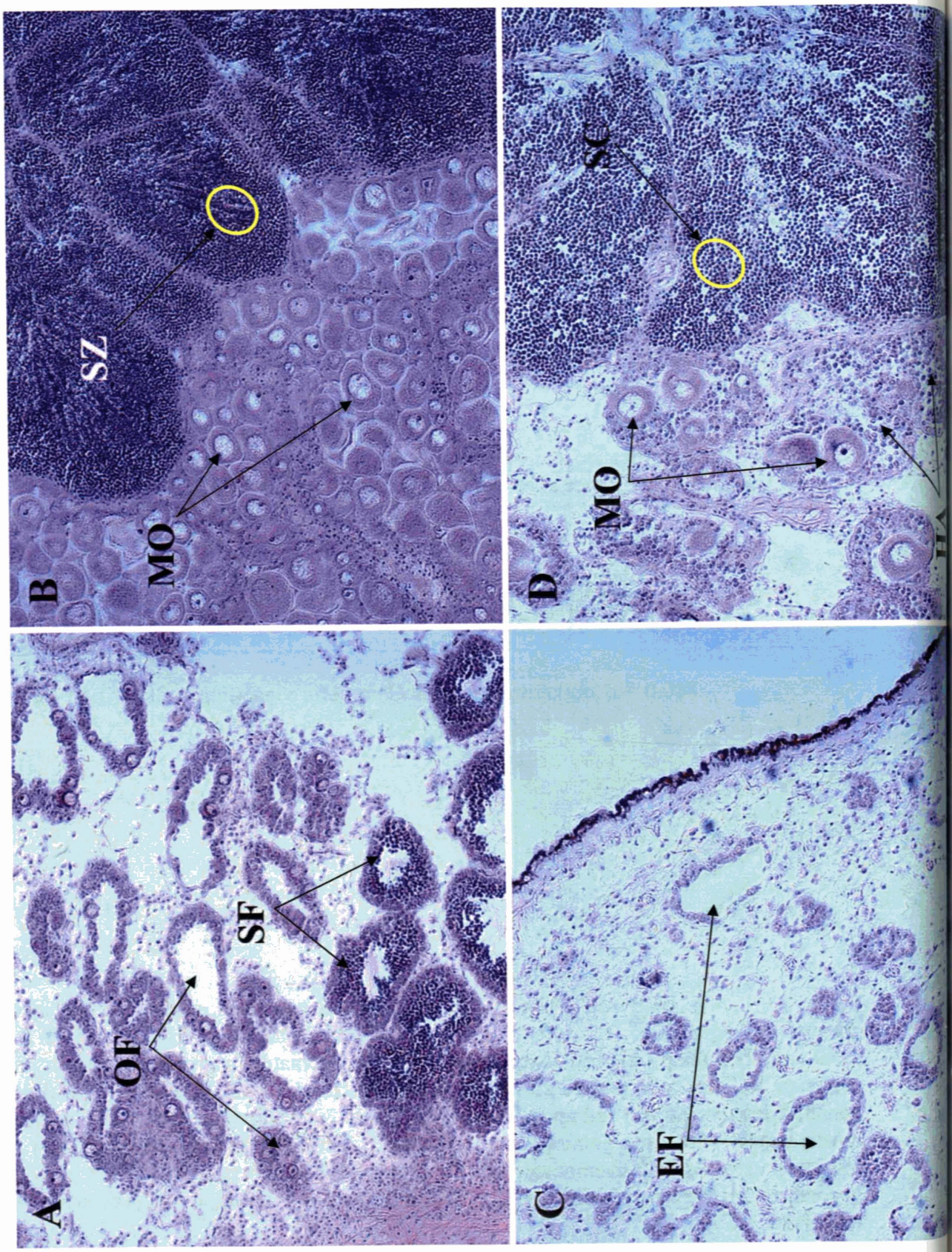
Note - ** Denotes significance after the Bonferroni correction, $\alpha = 0.008$

Table 3 - Results from t-tests conducted separately for both 2N and 3N bay scallops which compared the mean body indices observed for both May 31st and August 11th.

		Gonado-Somatic Index		Muscle Index		Rest Index	
Ploidy		Mean (SE)	P	Mean (SE)	P	Mean (SE)	P
2N	May 31	0.041 (.008)	0.0001**	0.270 (.015)	0.012*	0.689 (.017)	0.0003**
	Aug 11	0.185 (.007)		0.217 (.013)		0.599 (.014)	
3N	May 31	0.041 (.015)	0.0001**	0.320 (.020)	0.347	0.639 (.023)	0.038*
	Aug 11	0.128 (.011)		0.296 (.015)		0.576 (.018)	

Note - * denotes significance at $\alpha = 0.05$
** denotes significance after Bonferroni correction, $\alpha = 0.008$

Figure 13 – (A & B) typical 2N gonad development seen at deployment (A) and upon retrieval in August 2005 (B): note immature oocytes (OF) and sperm follicles (SF) in the immature 2N scallop (A), and mature oocytes (MO) and mature spermatozoa seen in the centre of follicles in a mature 2N scallop (B). In contrast (C&D) show a typical 3N gonad at deployment (C) and upon retrieval in August 2005 (D): note early follicles (EF), mostly empty with little gametic development (C), and large mature oocytes (MO) in follicles inundated with haemocytes (HA) and sperm development that does not proceed beyond the primary spermatocyte (SC) stage in a mature 3N scallop (D). The scale bar represents 225 μm.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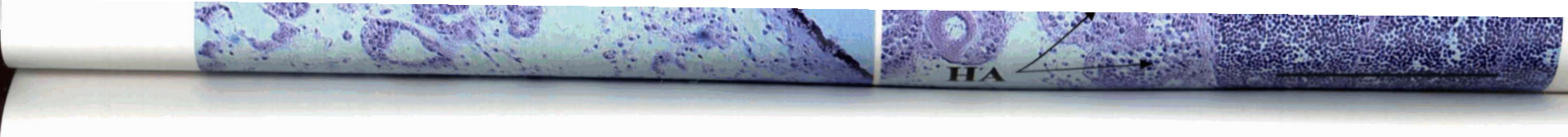

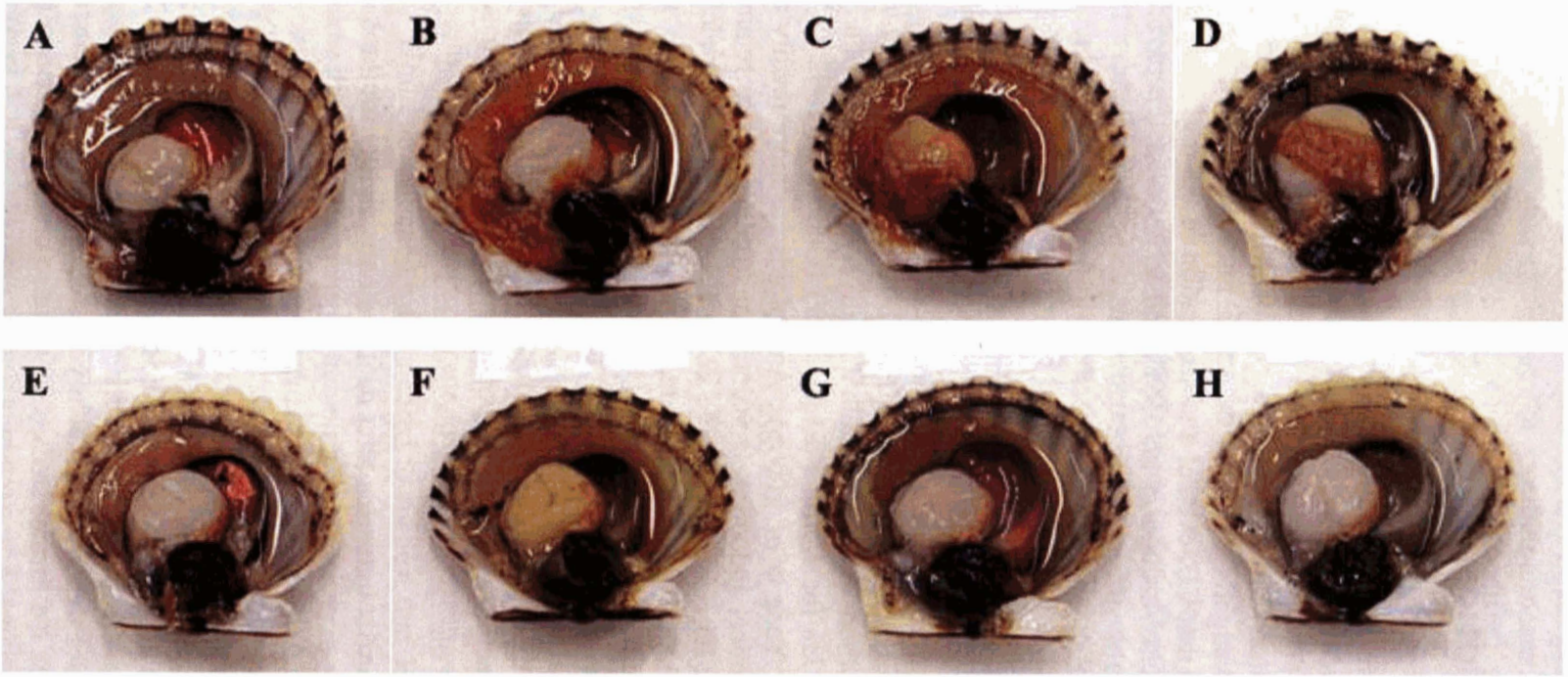

Figure 14 - Comparison of 2N (A-D) and 3N (E-H) gonads after 7 weeks of laboratory conditioning. The black membrane of 3N gonad E has been opened with scissors to reveal a bright orange gonad. While the orange color of the female portion of the gonad was visible in 3Ns, in general the thin black membrane typical of early gonad development was more evident in 3Ns.

DISCUSSION

TRIPLOID INDUCTION

Results from this report indicate that the optimal CB concentration for inducing 95-100% 3N bay scallops is 1 mg/l. While 0.5-1 mg/l of CB is routine for inducing 3Ns in other shellfish species (Beaumont and Fairbrother 1991), it was a 10 fold increase over the optimal induction value for bay scallops of 0.1 mg/l proposed by Tabarini (1984). Other authors investigating optimal induction rates for species within the *Argopecten* genus have shown that CB, even at a concentration of 0.5 mg/l, only produces ~56% 3N *A. ventricosus* (Ruiz-Verdugo *et al.* 1998) and 17% 3N *A. purpuratus* (Winkler *et al.* 1993). These results strongly suggest that the values proposed by Tabarini (1984) are well below the actual concentration necessary for optimal 3N production via exposure to CB.

Aneuploids seen in the control group as evidenced by the DNA index (< 1.5) of the "non-diploid" peak in ModFit LT outputs suggests that even in controls some partial chromosome retention is evident. This has been seen in 3N induction experiments with other species (*Crassostrea gigas, Haliotis diversicolor, Argopecten purpuratus,* and *Chlamys ferreri*) where controls contain a relatively large proportion of aneuploid nuclei in early larvae (47% for *Haliotis diversicolor*) (Guo *et al.* 1992; Winkler *et al.* 1993; Yang *et al.* 1998; Yang *et al.* 2000b). Aneuploids are more readily apparent when less than optimal chemical induction concentrations only partially retain the second polar body chromosome complement. While in the lab most aneuploids do not persist beyond the larval stages in either control or treatment groups, aneuploids in some species do persist through adulthood (*Crassostrea gigas*) and can sometimes even be found at fairly significant rates in the wild (*Mytilus sp.*) (Martinez-Exposito *et al.* 1992; Wang *et al.* 1999). This partial chromosome retention in some species (*C. gigas*) does not appear to noticeably effect their growth in comparison to 3Ns within the same treatment but less noticeable reproductive and physiological effects could alter their basic biology and with different chromosomes being present/absent in each aneuploid these changes could be highly variable.

Bay scallop 3N induction rates declined only slightly for the 1 mg/l treatment over the duration of the experiment. While our experiments only took small samples to assess the overall induction rate for the entire population, more work needs to be done examining the potential of reversion for single animals from the 3N state to a 2N one. Some studies seeing slight declines in 3N induction rates over time might attribute the decline to preferential mortality for 3Ns but it is possible that some animals actually fully revert to a viable 2N state. Some studies, by following individual 3N *C. gigas* and *C. ariakensis,* suggested that while reversion does occur it is incomplete and results in heteroploid mosaics that are undoubtedly as sterile as their 3N counterparts (Allen *et al.* 1996; Allen 2000). In further studies, it was also clear that this reversion is 2 to 3 times less likely to occur in 3Ns created by 4N x 2N crosses than it is to occur in 3Ns produced

by chemical induction (Eudeline and Allen 2000). The fact that reversion does occur in at least 2 species and that it has not been investigated in any other species of commercial interest is of concern. Future studies exploring 3Ns as either a means to improve growth or as a tool for introducing non-native or genetically modified shellfish into new environments should focus efforts on following individual 3Ns produced by various methods to better assess the risk for reversion from a sterile 3N state to a reproductively viable 2N state.

In general and as seen in this study, chemically induced 3Ns show dramatically reduced embryonic survival as compared to their 2N controls regardless of the species used (Baron *et al.* 1989; Guo and Allen 1994a; Nell *et al.* 1995; Stepto and Cook 1998; Supan *et al.* 2000; Jackson *et al.* 2003). Given the relatively high fecundity of shellfish, decreased survival associated with 3N induction via CB does not appear to be commercially prohibitive since most of the 3N *C. gigas* induced before 2000 were 3Ns produced in this manner (Nell 2002). Nonetheless, 4N by 2N crosses (i.e., "natural" triploids) eliminate the potential hazard of exposing early embryos to toxic chemicals and also improves the growth and survivability of "natural" 3Ns as compared to chemically induced 3Ns (Guo *et al.* 1996; Que *et al.* 2003). Given the commercial potential and the unavoidable regulatory concern surrounding reproductively viable 4Ns and the resulting 3Ns produced from crossing them with 2Ns, more work needs to be done to elucidate the potential impact that 4Ns may have on their surroundings if accidentally released. In-depth information surrounding current Canadian regulatory considerations for 3N shellfish can be found in Cogswell *et al.* (2005).

GROWTH, SURVIVAL AND GONADIC DEVELOPMENT

The intent of this study was not to examine relative disease resistance of 2N and 3N juvenile bay scallop. Nonetheless, scallops were sent to the DFO Shellfish Health Unit prior to being placed in the field for grow-out trials. As stated in the results section, of the 30 2Ns and 30 3Ns sent for full disease profiling 5 2Ns and 3Ns were infected with *Perkinsus karlssoni.* One month after field deployment (June 27th, 2005) survival was significantly reduced for both ploidies but more so for the 3N group where nearly 50% mortality occurred. While no evidence was gathered to suggest that 3Ns were any more susceptible to this non-lethal pathogen than 2Ns, reduced survival in the 3N group did suggest that 3Ns were more vulnerable to the combined stress associated with high stocking densities in the lab prior to deployment and the stress resulting from the transfer from the lab to the field. While there are conflicting results in regards to the overall disease resistance of 3Ns there is evidence in some species (*C. virginica*) to suggest the triploidy confers a negligible increase in disease resistance to the parasite *Haplosporidium nelsoni* (MSX) (Matthiessen and Davis 1992). In 3N *C. gigas* there appears to be no increased disease resistance to the parasite *Perkinsus marinus* (Meyers *et al.* 1991). In general however, field trials with 3N *C. gigas, M. edulis,* and *M. galloprovincialis* (Gordon King, pers. comm. 2003) have shown that under varying types of environmental stress (i.e., low productivity, starvation and summer mortality), 3Ns

show higher mortality rates in comparison to 2Ns (Davis 1988; Cheney *et al.* 2000; Brake *et al.* 2004). Despite evidence from these reports, there is not enough information yet available to suggest any advantage or disadvantage for using 3N shellfish for culture in terms of disease resistance and survival under varying culture conditions. To clarify this issue in-depth studies specifically examining disease resistance (i.e., multiple pathogen exposure) and mortality rates under various culture conditions need to be conducted for species of commercial interest.

While increased growth has been observed in most chemically-induced 3N species (Guo *et al.* 2001) shell parameters (i.e., length, height and width) did not significantly differ between 2N and 3N bay scallops over the duration of our observations. Nonetheless, condition indices (MI and GSI) did differ significantly between the treatment and control and over time for both ploidies. A decrease in 3N *Argopecten* (*irradians*, *ventricosus* and *purpuratus*) gonad condition as compared to their 2N counterparts has been previously observed (Tabarini 1984; Ruiz-Verdugo *et al.* 2001; Lohrmann and Von Brand 2005) but even within the *Argopecten* genus there is a discrepancy as to how triploidy affects both gametogenesis and energy reallocation. Our measurements showed a clearly significant decline in 2N muscle indices during gonadic proliferation. Tabarini (1984) suggested that these tissue growth differences between 2Ns and 3Ns are due to the way 2Ns sequester energy reserves, mostly in the form of glycogen from primarily adductor muscle, to account for the drastic increase in gonadic indices (Barber and Blake 1981); however, there is even some disagreement as to the nature of how energy reserves are partitioned in 2N scallops with some researchers suggesting that protein, not glycogen, is the energy reserve primarily partitioned during gametogenesis (Epp *et al.* 1988). 3N bay scallops, because gametogenesis is severely stunted, have no need to sequester these reserves from somatic tissue and likely account for their reduced gonadic proliferation via ingested food as seen in *A. purpuratus* (Lohrmann and Von Brand 2005). Histological examination of *A. irradians* gametic development conducted in our lab appeared very similar to both *A. purpuratus* and *Chlamys nobilis* (another hermaphroditic scallop species) gametic development and all appear to maintain hermaphroditic characterisitics despite the extra chromosome set (Komaru and Wada 1989). The effects of 3N induction on gametogenesis and sex-ratio are species specific and highly variable. For example, chemically induced 3N *Mytilus sp.* are nearly all male (Kiyomoto *et al.* 1996; Brake *et al.* 2004) and various deviations from the typical sex ratio for the species of shellfish being examined are commonly observed (Allen *et al.* 1986; Guo and Allen 1994a; 1998).

The phenomenon of increased growth for 3N's has been observed in many species of commercially important shellfish and has been largely attributed to, among other factors, the reallocation of glycogen reserves to somatic tissue at a time when 2N's are storing these reserves for gonadic maturation (Allen and Downing 1986). As is seen in some species 3N individuals show improved growth before the onset of maturation but this is not always the only cause for improved growth. As well, increased heterozygosity among 3N's due to an additional set of chromosomes seems to be a contributing factor to their increased growth. There is evidence to suggest that increased heterozygosity in 3N's (as evidenced by polar body II, polar body I and 4N x 2N matings) results in a positive correlational increase in overall meat weight (Wang *et al.* 2002). Lastly, similar

to what has been seen in Atlantic salmon (*Salmo salar*), triploidy results in larger overall cell size to accommodate their elevated nuclear volume (Benfey *et al.* 1984). This has not resulted however in larger salmon because unlike shellfish, finfish posses a homeostatic mechanism which reduces cell number thus resulting in a 3N animal of similar proportions to their 2N counterparts. In shellfish (notably clams – (Guo and Allen 1994a)), this regulative mechanism does not appear to exist and the resulting increase in cell size from 3N induction produces inherently larger animals. It is likely that not just any one of these factors (energy reallocation, heterozygosity and gigantism) contributes solely to the elevated growth rates observed in various species of shellfish but that each factor contributes in varying degrees in accordance with environmental factors and species specific developmental stages (Beaumont and Fairbrother 1991).

SUMMARY

Triploid bay scallops are easily produced by recently developed chemical induction techniques but given the toxicity of these chemicals and that induction is nearly always less than 100%, focus is shifting to the indirect method of 3N production via 4N x 2N crosses. Histological analysis of gametic development in bay scallops shows what appear to be mature oocytes. If techniques can be developed and modified to fertilize these eggs with haploid sperm and retain either the first or the second polar body then viable 4N offspring might be feasible.

Increased adductor size in 3N bay scallops (~8%) in comparison to 2Ns might make them an attractive alternative to 2Ns. If however the market value of the animal is gauged in shell height or the animal is sold whole, than this slight increase in adductor weight might be of limited value. Our observations certainly did not compare with the 73% increase in adductor weight in 3N bay scallops observed by Tabarini (1984). The reasons for this difference are unclear but certainly a shortened growing season and very different culture conditions likely contributed; however, given the unusually high early mortality rates likely attributable to handling and field transfer, our results are not definitive and further study is required.

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APPENDICIES

1. Instructions for making Propidium iodide nuclear staining solution and detailed protocols for preparing samples for ploidy analysis via flow cytometry (a-c).
2. Spawn II larval and juvenile sampling regime.
3. Juvenile rearing and thinning schedule.
4. Formula for Davidson's solution.
5. Preparation and staining procedure for scallop sections. Short description of disease profile methodology.

Appendix 1: Protocols for preparing samples for ploidy analysis via flow cytometry.

To make 200 ml of Propidium iodide stock solution, mix together the following (suggest using a 200ml volumetric flask):

0.01 g Propidium iodide (Sigma #P4170)
0.2 g Sodium citrate
20 ml DMSO
179.8 ml ddH_2O

Store refrigerated in a dark container, protected from light.

a) Staining procedure for larvae

1. Collect several hundred larvae in a small microcentrifuge tube (e.g., 1.7 ml), enough to form a small pellet of concentrated larvae in the bottom of the tube.
2. Remove supernatant seawater, leaving just the pellet of larvae.
3. Add 0.5 ml of Propidium iodide solution.
4. Close the lid of tube and shake vigorously on a vortex mixer for ~20 seconds.
5. Incubate in the dark at room temperature for at lease 10 minutes.
6. Shake again on a vortex mixer for ~10 seconds.
7. Remove solution from the 1.7 ml tube with 1 cc syringe and inject through 20µm filcon filter and collect filtrate in a flow cytometer tube.
8. Run the sample on low setting of the flow cytometer.

b) Staining procedure for juveniles (<500µm shell height)

1. Place an individual in the bottom of microcentrifuge tube and grind with a hand held pellet pestle (Sigma #Z359971-1EA).
2. Remove some tissue to form a small homogenized pellet on the bottom of the tube.
3. Add 0.5 ml of Propidium iodide solution.
4. Close the lid of tube, and shake on the high setting of a vortex mixer for ~30 seconds.
5. Incubate in the dark at room temperature for at least 10 minutes.
6. (If the sample must be run at a later time, freeze the sample now at -70°C).
7. Shake again on a vortex mixer for ~20 seconds.
8. Remove solution from the 1.7 ml tube with 1 cc syringe and inject through 20µm filcon filter and collect filtrate in a flow cytometer tube.
9. Run the sample on the low setting of the flow cytometer.

c) Staining procedure for adults (>1 cm shell height)

1. Cut a small amount of tissue into small pieces, as finely as possible.
2. Place tissue in a small microcentrifuge tube (e.g., 1.7 ml size tube), enough to form a small pellet of concentrated tissue in the bottom of the tube.
3. Remove the supernatant seawater, leaving just the pellet of tissue.
4. Add 0.5 ml of Propidium iodide solution.

5. Close the lid of the tube, and shake vigorously on a vortex mixer for ~30 seconds.
6. Incubate in the dark at room temperature for at least 10 minutes.
7. (If the sample must be run at a later time, freeze the sample now at -70°C).
8. Shake again on a vortex mixer for ~20 seconds.
9. Remove from the 1.7 ml tube with a 1 cc syringe and inject through a 20μm filcon filter and collect the filtrate in a flow cytometer tube.
10. Run the sample on the flow cytometer.

Appendix 2: Spawn II larval and juvenile sampling regime.

Date	Days Post-Fert	Treatment	Samples taken	Purpose of Sample
April 15th, 2004	0	Control	1 each	Initial population size of treatment.
		0.75 mg/l CB		
		1 mg/l CB		
April 16th, 2004	1	Control	1 each	Developmental assessment after 24 hours.
		0.75 mg/l CB		
		1 mg/l CB		
April 19th, 2004	4	Control	3 each	Initial ploidy induction rate assessments via flow cytometry. D-larvae survival and development.
		0.75 mg/l CB (not followed beyond day 4)		
		1 mg/l CB		
April 21st, 23rd, 28th	6, 8 and 13	Control	10 indiv/treat	Shell height assessment.
		1 mg/l CB		
April 21st, 2004	6	Control	2 each	Survival
		1 mg/l CB		
April 28th, 2004	13	Control	3	Survival (only 1 sample from CB treatment because of low numbers).
		1 mg/l CB	1	
May 5th, 2004	20	Control	1 each	Animals setting, sample taken for ploidy analysis.
		1 mg/l CB		
May 17th, 2004	32	Control	1 vial/treat	Samples from the non-set and set portions for ploidy analysis.
		1 mg/l CB		
July 29th, 2004	105	1 mg/l CB	40 indiv	Individual ploidy analysis.
Sept 2nd, Oct 21st, Jan 6th 2005, Mar 14th, Apr 12th, May 12th, May 31st	140 - 410	Control	All scallops counted in both control and treatment.	Scallops periodically thinned.
		1 mg/l CB		
April 18th, 2005	368	Control	30 indiv/treat	Assessing ploidy, whole wet weight, height, length, width and histology/disease profiling.
		1 mg/l CB		

Appendix 3: Juvenile rearing and thinning schedule.

Date	#/net	Mesh Size (inches)	Density (#/sq·inch)	Total Animals/Ploidy
Sept 2, 2004	500-600	0.24" (6 mm)	~2.5 - 3	3200
Oct 24, 2004	300	0.24"	~1.5	1800
Jan 6, 2005	152	0.24"	~0.8	912
Mar 16, 2005	110	0.47" (12 mm)	~0.6	660
April 12, 2005	80	0.47"	~0.4	480
April 19, 2005	75	0.47"	~0.4	450
May 12, 2005	60	0.47"	~0.3	360

Appendix 4: Formula for 4L of Davidson's solution.

400ml Glycerin (or glycerol)
800ml 37-40% Formaldehyde
1200ml 95% Ethanol
400ml Glacial Acetic Acid
1200ml Reverse Osmosis Water

* Store at ~ 4^0C and lasts for approximately 1 month.

Appendix 5: A short description of disease profile methodology.

1. Single 6 μm thick sections from each animal were assessed during disease screening. Sections each contain a bit of digestive gland, gill, gonad, adductor muscle and kidney.
2. Slides were first "quick scanned" under the microscope - observed with a 4x objective. Ratings were given to gut content, infiltration (haemocyte activity), diabetes's (haemocytes across epithelial walls) and metaplasia (flattening of the tubule epithelia).
3. The animals were then "full scanned"- observed with a 10x objective. During this process any parasites or disease agents observed were documented by presence/absence. Tissue necrosis and/or tissue breakdown was also documented. A sub-sample of animals was cross-checked by a second diagnostician for QAQC purposes.

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