Investigations with Triploid Atlantic Sea Scallops, *Placopecten magellanicus*, at the Bedford Institute of Oceanography, 2000-2003

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INVESTIGATIONS WITH TRIPLOID ATLANTIC SEA SCALLOPS, *PLACOPECTEN MAGELLANICUS*, AT THE BEDFORD INSTITUTE OF OCEANOGRAPHY, 2000-2003

by

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TABLE OF CONTENTS

ABSTRACT

The use of triploid bivalves has become established in the aquaculture industry as a viable means of improving growth rates and product quality, but few attempts have been made to apply this technology to the sea scallop, *Placopecten magellanicus*. A series of trials were conducted to study the effectiveness of three different triploidy induction agents (Cytochalasin B (CB), 6-dimethylaminopurine (6-DMAP), and thermal shock) for use with sea scallops. CB application routinely yielded higher proportions of triploids (up to 98%) than any other mechanism. The use of 6-DMAP produced some triploids, but most were deformed and mortality was high with none surviving beyond 12 days of age. Heat shock was essentially ineffective in producing triploids, with all putative triploids disappearing from the populations early in development. Suppression of meiosis I with CB did not produce as many triploids (35% - 60%) as meiosis II blocking (51 - 88%). Experiments conducted to determine the optimum combination of CB concentration (0.25 mg/l and 0.50 mg/l) and incubation temperature (6°C and 14°C) revealed that the highest percentages of triploids were obtained when embryos were incubated for 20 minutes in a CB concentration of 0.50 mg/l at 14°C. CB concentration played a significant role in the success of triploidy induction at 14°C, but did not have much of an effect at 6°C. Larval growth and survival rates were low in all treatments, but evidence is presented to suggest that *Placopecten magellanicus* triploids have the potential to grow at faster rates and may have larger cells than diploids, thereby supporting a hypothesis of polyploid gigantism.

RÉSUMÉ

L'industrie aquacole utilise désormais couramment les bivalves triploïdes afin d'améliorer les taux de croissance et la qualité du produit de façon économique. Peu d'essais cependant ont été effectués pour appliquer cette technologie au pétoncle géant, *Placopecten magellanicus*. Une série d'essais ont été entrepris pour étudier l'efficacité de trois agents d'induction de la triploïdie (Cytochalasine B (CB), 6-dimethylaminopurine (6-DMAP), et choc thermique) en vue de leur utilisation sur le pétoncle géant. L'utilisation de CB a constamment donné des proportions de larves triploïdes plus élevées (jusqu'à 98%) que tout autre agent; l'utilisation de 6-DMAP a permis de produire des larves triploïdes mais la plupart étaient déformées et ont subi des taux de mortalité élevés et aucune larve n'a survécu après 12 jours. Les chocs thermiques se sont avérés inefficaces pour produire des larves triploïdes, en raison de la disparition des larves triploïdes tôt dans leur développement. La suppression de la méiose I avec la CB n'a pas produit autant de larves triploïdes (35% - 60%) que le blocage de la méiose II (51%-88%). Les expériences réalisées pour optimiser la combinaison de la concentration de CB (0.25 mg/l et 0.50 mg/l) et de la température d'incubation (6°C et 14°C) ont permis de montrer que les pourcentages les plus élevés de larves triploïdes ont été obtenus en incubant les embryons pendant 20 mn à une concentration de CB de 0.50 mg/l et à une température de 14°C. La concentration de CB a joué un rôle significatif quant au succès de l'induction de la triploïdie à une température de 14°C, mais n'a pas eu d'effet à une température de 6°C. La croissance larvaire et les taux de survie ont été faibles dans tous les traitements, mais on observe des signes suggérant que les larves triploïdes de *Placopecten magellanicus* peuvent croître à des taux plus élevés et pourraient présenter des cellules plus grandes que celles des larves diploïdes, supportant ainsi l'hypothèse de gigantisme polyploïde.

INTRODUCTION

The successful commercial culture of the Atlantic sea scallop, *Placopecten magellanicus*, faces many challenges, some of which can be addressed by the application of biotechnological advancements. One area of investigation in the culture of marine bivalves that has received a lot of attention is the development of triploid shellfish. Instead of the usual two sets of chromosomes that are normally found in diploid organisms, triploids have three sets of chromosomes, a feature that gives them special qualities that are of benefit to the aquaculture industry.

Much of the value of triploids stems from the fact that they are essentially sterile, a condition that arises because homologous chromosomes presumably cannot synapse at meiosis in the germ cells of triploids (Beaumont and Fairbrother, 1991). Sterility imparts several benefits that can be exploited by the aquaculturist. Principal among these is that sterile animals do not allocate energy to developing their gonads; therefore more energy is available for somatic growth and the animals grow at greater rates (Ruiz-Verdugo *et al.*, 2001). The second benefit of culturing sterile triploids is that they usually have better meat quality than diploids, since the energy stored in adductor muscle as glycogen is not redirected to gonads during reproductive season. Higher levels of glycogen in shellfish has been linked to superior quality and better taste, and in bivalves such as oysters the gonad is located throughout the somatic tissue rendering the product soft and unpalatable (Allen and Downing, 1991). Finally, the possibility of introducing cultured sterile triploid animals into areas where they do not otherwise occur holds promise for the expansion of aquaculture into new regions without the concerns of local ecosystem perturbations (Beaumont, 2000).

Since increased growth rate equates with faster time to market, this feature of triploidy is of great interest to the aquaculturist. In most molluscan species studied to date, triploids grow at significantly greater rates than diploids (e.g., *Crassostrea gigas* (Allen and Downing, 1986; Wang *et al.*, 2002); *Crassostrea virginica* (Stanley *et al.*, 1984; Matthiessen and Davis, 1992); *Saccostrea glomerata* (Nell *et al.*, 1994); *Argopecten irradians* (Tabarini, 1984); *Argopecten ventricosus* (Ruiz-Verdugo *et al.*, 2000); *Chlamys farreri* (Yang *et al.*, 2000). In addition to sterility, there are two other factors that may contribute to this phenomenon. Triploids are theoretically more heterozygous than diploids and as such demonstrate the condition of "larval vigor" in which greater growth and higher overall fitness are reported (Stanley *et al.*, 1984; Mason *et al.*, 1988; Wang *et al.*, 2002). The third hypothesis, known as polyploid gigantism, relates to the size of triploid cells; since triploid nuclei have 50% more DNA than diploids, the cell's cytoplasm is expected to be proportionally larger in order to support the larger nucleus and maintain an optimum cytoplasm/nucleus ratio (Guo and Allen, 1994a; Wang *et al.*, 2002).

Triploids are produced by a variety of methods which involve the blocking of polar body formation in the egg maturation divisions of either meiosis I or meiosis II. Treatment with either chemical or physical (temperature or pressure)

shocks at these developmental phases can result in the retention of an extra set of chromosomes in the fertilized egg and subsequent "chromosome doubling" (Beaumont and Fairbrother, 1991). Of the chemicals utilized to block polar body formation, the fungal metabolite microfilament inhibitor Cytochalasin B (CB) has proven to be most effective (Scarpa *et al.*, 1994), although other agents like 6-dimethylaminopurine (6-DMAP) and caffeine have been used with varying degrees of success. 6-DMAP in particular holds promise for future use as a triploid induction agent since it is not as toxic as CB (Desrosiers *et al.* 1993), and there are health concerns related to the use of CB in the food industry (Guo *et al.*, 1994). The most successful, and safest, method of producing triploids however is by crossing tetraploids with diploids to produce 100% triploid offspring (Guo and Allen, 1994b; Nell, 2002).

The development of triploid technology has proven to be very beneficial to the bivalve aquaculture industry, with a noteworthy example being the US West Coast Pacific oyster sector where over 30% of the total product is now comprised of triploids (Nell, 2002). In Atlantic Canada, the culture of the sea scallop is at a much less developed state, and few trials have been conducted on the feasibility of producing triploid sea scallops (Desrosiers *et al.* 1993). More research into the production of triploid sea scallops is required if sea scallop culture is to advance and develop into a viable, sustainable industry. This document outlines the work that has been conducted with triploid sea scallops at the Bedford Institute of Oceanography between October 2000 and December 2002, which was geared to develop and evaluate techniques for the commercial production of triploid and tetraploid sea scallops. The emphasis of this research was on triploidy induction techniques; experiments were conducted on determining which meiotic division should be targeted, and different types of chemical agents were tested along with evaluation of the effectiveness of heat shock as a polar body blocking mechanism.

MATERIALS AND METHODS

EXPERIMENT 1: POLAR BODY BLOCKING TRIAL, FALL 2000

The first experiment was designed to determine if Cytochalasin B (CB) is effective as an agent for inducing triploidy in sea scallops, and to study the effects of using CB to block the formation of polar bodies 1 and 2 (PB1 and PB2). In this initial trial, it was decided to try a CB concentration of 0.50 mg/l and an incubation temperature of 14°C as triploidy induction parameters. Three replicate spawning lots were produced on October 14, 2000, each of which was divided into 3 groups: a control, a treatment to block polar body 1 formation, and another to block polar body 2.

In the first spawning lot, eggs from 1 female were fertilized with sperm from 2 males in 4 litres of filtered seawater. To block polar body 1 formation, the development of the embryos was closely monitored to observe the first appearance of PB1. This effort was facilitated by monitoring the development times of a "test spawn", which was fertilized 12 minutes before the main spawn using gametes from the same parents, enabling accurate prediction of the expected time of PB1 release. At 42 minutes post-fertilization, approximately 5-10% of the embryos had released PB1, and CB was added at this time. To block polar body 2, CB was added to a second group of embryos when about 50% of them had extruded PB1, at 54 minutes post-fertilization (see Table 1).

In both the PB1 and PB2 groups, the embryos were gently mixed during CB exposure with a sterile plunger to keep them suspended in the water column, and after 20 minutes the CB was removed by pouring the embryos over a 20 µm Nitex screen and rinsing them with seawater. The embryos were then transferred into 300 litre insulated tanks (14°C) for development to D-stage and subsequent rearing. Control treatment larvae were transferred into an identical tank at the same time.

The triploidy induction process was repeated in two other spawning lots (see Table 1 for induction parameters). In Lot 2, the broodstock consisted of 3 females and 3 males, and in Lot 3, the gametes were pooled from 5 females and 3 males. Thus, a total of 9 treatments were prepared; three controls (Control 1, 2, and 3), three different fertilizations in which polar body 1 was blocked (PB1-1, 2, and 3), and three trials to block polar body 2 (PB2-1, 2, and 3). All larvae were fed, changed, and measured at regular intervals, and samples were taken from each treatment for ploidy analysis on Days 3, 15, and 54. Ploidy analyses were conducted according to the procedures outlined in Appendix 3a and Appendix 4. All larvae were transferred to downwellers on Day 55, when they had started to show signs of metamorphosis.

EXPERIMENT 2: INCUBATION TEMPERATURE AND CB CONCENTRATION TRIAL, SUMMER 2001

The results from Experiment 1 demonstrated that Cytochalasin B was an effective agent for blocking PB2 release. Therefore, Experiment 2 was designed to study the interactions between Cytochalasin B concentration and incubation temperature on induction of triploidy via PB2 blocking. The two-factor experimental design included two levels of Cytochalasin B (0.25 mg/l & 0.50 mg/l), and 2 incubation temperatures (6°C & 14°C), as well as a control treatment for each incubation temperature. Triploidy induction was performed on two separate spawning lots on June 22, 2001. In the first lot, eggs from one female were fertilized with sperm from one male, while two females and two males were used in the second lot.

As in Experiment 1, CB was added to a treatment when about 50% of the embryos exhibited signs of PB1 release, and exposure time to CB ranged from 20 to 26 minutes (consult Table 1 for CB application times and durations). Embryos were kept suspended in the CB bath by gentle mixing, and were screened and rinsed on a 20 µm Nitex screen to remove the CB. All embryos were reared at 14°C in 300 litre insulated tanks, and were fed, changed, and measured regularly. Larval ploidy analyses were conducted for all treatments on Days 4, 11, 19, & 55, according to the procedures outlined in Appendix 3a and Appendix 4.

EXPERIMENT 3: "OPTIMUM INDUCTION CONDITIONS" TRIAL #1, FALL 2001

The most successful conditions for inducing triploidy, as identified in Experiment 2, were used in Experiment 3 to produce large numbers of triploid sea scallop larvae. On October 30, 2001, eggs from 4 females were fertilized with sperm from 3 males, and were incubated in 2 litres of filtered seawater in a 5 litre bucket at 14°C. Embryos were regularly checked for polar body formation, and Cytochalasin B (final concentration of 0.50 mg l^{-1}) was added when approximately 50% of the embryos had extruded the first polar body, at 60 minutes post-fertilization. The embryos were gently stirred to keep them suspended. After 21 minutes exposure, the Cytochalasin B was removed by first pouring the embryos over a 20 µm Nitex screen. The embryos were then rinsed with seawater and suspended in a 2 litre bath of 0.01% DMSO in seawater for 15 minutes (to remove remaining traces of Cytochalasin B), after which time they were again poured over a 20 µm Nitex screen and rinsed with seawater. The embryos were then transferred into a 1000 litre insulated tank (14°C) for rearing.

A control group of larvae was also produced, being fertilized from the same batch of parents at the same time as the Cytochalasin B treatment group ("CB-1"). As a sham treatment, these larvae were treated with DMSO in the same manner as the CB-1 larvae. Both groups of larvae were changed, fed, and measured approximately every two days, and samples were taken for ploidy analysis on days 3, 7, 9, 15, 17, 21, 27, and 41. Ploidy analyses procedures are outlined in Appendix 3a and Appendix 4.

EXPERIMENT 4: "OPTIMUM INDUCTION CONDITIONS" TRIAL #2, WINTER 2002

A second attempt to produce large numbers of competent triploid sea scallops was made on March 12, 2002, in which one control group and two Cytochalasin B treatment groups were prepared. The same induction conditions used in Experiment 3 (0.50 mg l^{-1} CB, 14°C incubation temperature) were reproduced here. The first triploid induction group (CB-1) was treated with Cytochalasin B 69 minutes after fertilization, and a second group (CB-2) was treated 54 minutes post-fertilization (see Table 1). Exposure time to Cytochalasin B was 20 minutes in both cases before rinsing and DMSO exposure. The control group was treated with DMSO at the same time as the CB-1 group was treated.

After transfer of the CB-1 and CB-2 larvae to their respective 1000 litre insulated tanks at an optimum stocking density (~10 larvae/ml), it was discovered that there was a surplus of larvae. These larvae were combined together as group "CB-MIX", and placed in a 300 litre insulated tank. All groups of larvae were changed, fed, and measured approximately every two days, and samples were taken for ploidy analysis at regular intervals. The CB-2 group larvae were all dead by Day 20, and by Day 38 the low numbers of larvae remaining in the CB-1 and CB-MIX groups warranted that they be combined in a single tank for subsequent rearing. The ploidy composition of this group was measured on Day 50.

EXPERIMENT 5: "OPTIMUM INDUCTION CONDITIONS" TRIAL #3, FALL 2002

A third attempt to produce large numbers of competent triploid sea scallops was made on October 12, 2002, using the same procedures as those defined in Experiments 3 and 4. In this trial, the parent broodstock were obtained from Mahone Bay, Nova Scotia, and brought into the lab just four days prior to spawning. In addition to a control group, triploid induction was conducted on two replicate spawning lots (CB-1 and CB-2). Approximately 14 million eggs were treated with Cytochalasin B at 14°C for 22 minutes in the CB-1 treatment, and another 14 million eggs were treated with CB for 20 minutes in the CB-2 group (Table 1). All eggs were rinsed with seawater and DMSO as described above. Larvae were changed, fed, and measured at regular intervals, and samples were taken for ploidy analysis on days 2, 3, 8, 15, 22, 29, 35, and 45 (see procedures in Appendix 3a and Appendix 4).

A subsequent spawn was made on October 29, 2002, using gametes from the same group of parents. The CB-3 and CB-4 groups, containing 10 million and 8 million eggs respectively, were treated with CB using the same procedures as those used in the October 12 spawn (see Table 1 for details). A control group was also prepared as described above. All larvae were fed and measured at regular intervals, and ploidy measurements were made on days 14, 21, and 51.

As a result of the trials conducted to date, the procedures for conducting triploid induction with Cytochalasin B in sea scallops were well refined and standardised, and are reproduced in itemised form in Appendix 1.

EXPERIMENT 6: HEAT SHOCK TRIAL #1, SUMMER 2001

Experiment 6 was conducted to determine if triploidy can be induced in sea scallops via the application of a thermal shock to block Polar Body 2 release. On June 22, 2001, eggs from 2 females were fertilized with sperm from 2 males, and allowed to develop at 14°C until approximately 50% of embryos had extruded Polar Body 1 (64 minutes; see Table 1). Heat shock was applied by transferring the embryos into a container of 22°C seawater for 12 minutes, after which they were placed into a 1000 litre insulated tank (14°C) for subsequent incubation and rearing. An untreated control treatment was also prepared, in which the embryos underwent early development at 14°C. Larvae were fed and measured at regular intervals, and ploidy measurements were made on days 4, 11, 19, and 55 (see procedures in Appendix 3a and Appendix 4).

EXPERIMENT 7: HEAT SHOCK TRIAL #2, FALL 2001

Experiment 7 was conducted to study the effects of using different levels of heat shock (5°C, 10°C, and 15°C above ambient temperature) on triploid induction in sea scallops. On October 30, 2001, gametes from four females and three males were used in this trial, and the fertilised embryos were incubated in a series of 5 litre buckets at 15°C. In the 20°C heat shock group, approximately 4 million embryos were apportioned between two replicate buckets, and heated seawater was added to bring the temperature up to 20°C when approximately 50% of embryos had released PB1 (see Table 1). The temperature exposure lasted for 10 minutes, at which time the embryos were transferred into two 300 litre insulated tanks for rearing, at 14°C. This procedure was repeated for the two replicate lots of the 25°C and 30°C treatments. The control treatment in this experiment was regarded as the Control for Experiment 3, which was spawned from the same parents on the same day and was not subjected to any thermal shock during development. Ploidy measurements were made on days 4, 11, 19, and 55 (see procedures in Appendix 3a and Appendix 4).

EXPERIMENT 8: 6-DMAP TRIAL #1, SPRING 2002

On May 24, 2002, an experiment was started to test the use of 6-DMAP as an agent for inducing triploidy in sea scallops. Two different concentrations of 6-DMAP were used (200 µM and 400 µM), with two replicate spawning lots for each concentration treatment. After fertilisation, embryos were incubated in a 5 litre bucket at 12°C until polar body 1 formation was observed in about 50% of the embryos, at which time 6-DMAP was added (see Table 1). The buckets were thoroughly stirred to dissolve the 6-DMAP powder and keep the embryos suspended. After approximately 20 minutes exposure (times varied slightly; see

Table 1), the embryos were poured over a 20 µm Nitex screen and rinsed with filtered seawater to remove the chemical. Embryos were transferred to 300 litre insulated tanks for subsequent rearing at 14°C. An untreated Control group of larvae was reared in a 300 litre insulated tank. Samples were taken for ploidy analysis on Day 4.

EXPERIMENT 9: 6-DMAP TRIAL #2, FALL 2002

A second attempt to test the use of 6-DMAP as an agent for inducing triploidy in sea scallops was made on October 29, 2002, using procedures similar to those defined in Experiment 8. In this trial, only one concentration of 6-DMAP was used (400 µM), and no replicate spawning lots were produced. Eggs from 2 females were fertilised with sperm from 6 males, and were incubated at 14°C for 64 minutes before addition of 6-DMAP. The embryos were gently mixed with a sterile plunger for 15 minutes before they were poured over a 20 µm Nitex screen and rinsed with filtered seawater to remove all traces of the 6-DMAP. Embryos were reared at 14°C in a 300 litre insulated tank until they were three days old, when samples were taken for ploidy analysis.

RESULTS

The experimental design parameters for all ploidy induction trials conducted in this study are summarized in Table 1, along with the percentage of triploid larvae found in samples taken when the larvae reached D-stage, usually at three to four days of age.

Table 1. Summary of experimental parameters for sea scallop triploidy induction experiments conducted at the Bedford Institute of Oceanography, 2000-2002.

Exp. #	Treatment ID[1]	Spawning Date	Triploidy Induction Agent	Conc.	Incubation Temp. (°C)	Treatment Start Time[2] (min)	Duration[3] (min)	Initial 3n%[4]
1	PB1-1	Oct 14/00	CB	0.50 mg/l	14	42	20	44
	PB1-2	Oct 14/00	CB	0.50 mg/l	14	35	21	35
	PB1-3	Oct 14/00	CB	0.50 mg/l	14	34	24	60
	PB2-1	Oct 14/00	CB	0.50 mg/l	14	54	20	59
	PB2-2	Oct 14/00	CB	0.50 mg/l	14	49	21	51
	PB2-3	Oct 14/00	CB	0.50 mg/l	14	54	21	88
2	L1 6 25	Jun 22/01	CB	0.25 mg/l	6	73	26	25
	L1 14 25	Jun 22/01	CB	0.25 mg/l	14	63	26	61
	L1 6 50	Jun 22/01	CB	0.50 mg/l	6	73	26	24
	L1 14 50	Jun 22/01	CB	0.50 mg/l	14	63	26	59
	L2 6 25	Jun 22/01	CB	0.25 mg/l	6	48	20	13
	L2 14 25	Jun 22/01	CB	0.25 mg/l	14	39	20	39
	L2 6 50	Jun 22/01	CB	0.50 mg/l	6	48	20	16
	L2 14 50	Jun 22/01	CB	0.50 mg/l	14	39	20	61
3	CB-1	Oct 30/01	CB	0.50 mg/l	14	60	21	98
4	CB-1	Mar 12/02	CB	0.50 mg/l	14	69	20	85
	CB-2	Mar 12/02	CB	0.50 mg/l	14	54	20	95
5	CB-1	Oct 1/02	CB	0.50 mg/l	14	69	22	56
	CB-2	Oct 1/02	CB	0.50 mg/l	14	51	20	93
	CB-3	Oct 29/02	CB	0.50 mg/l	14	65	20	24
	CB-4	Oct 29/02	CB	0.50 mg/l	14	60	20	45
6	Heat-22	Jun 22/01	Heat Shock	-	22	64	12	36
7	Heat-20-1	Oct 30/01	Heat Shock	-	20	63	10	0
	Heat-20-2	Oct 30/01	Heat Shock	-	20	63	10	29
	Heat-25-1	Oct 30/01	Heat Shock	-	25	65	10	26
	Heat-25-2	Oct 30/01	Heat Shock	-	25	65	10	31
	Heat-30-1	Oct 30/01	Heat Shock	-	30	65	10	-
	Heat-30-2	Oct 30/01	Heat Shock	-	30	65	10	-
8	DMAP-1A	May 24/02	6-DMAP	200µM	10	86	23	52
	DMAP-1B	May 24/02	6-DMAP	200µM	10	72	21	77
	DMAP-2A	May 24/02	6-DMAP	400µM	10	65	25	60
	DMAP-2B	May 24/02	6-DMAP	400µM	10	71	20	94
9	DMAP-1	Oct 29/02	6-DMAP	400µM	14	64	15	70

[1] Identification code for each treatment group
[2] The time at which treatment was started, measured as minutes after fertilization
[3] The duration of exposure to the treatment agent, measured in minutes
[4] The initial proportion of the population that was triploid, measured when larvae reached D-stage

EXPERIMENT 1: POLAR BODY BLOCKING TRIAL, FALL 2000

The ploidy data from Experiment 1 are summarized in Table 2 and Figs. 1-3. In all three spawning lots, there was a high proportion of triploids in the control groups on Day 3, ranging between 13% and 41% (Fig. 1). In Lot 1 and Lot 2, there was a slight increase in the percentage of triploids throughout the duration of the experiment that may have been the result of increased mortality of the diploid component of the population. In Lot 3, the proportion of triploids declined over time, with none left by Day 55.

In the PB1 blocking groups, initial triploid percentages were also high, ranging from 35% in Lot 2 to 60% in Lot 3. When sampled on Day 15 these numbers had increased in all three lots, a trend that coincided with the complete loss of the aneuploids and tetraploids in the populations. The proportion of triploids in the PB1 treatment populations stayed relatively constant between Day 15 and Day 54.

The highest level of triploidy was found in the PB2 blocking groups. On Day 3 51% of the larvae in Lot 2 were triploid, as were 59% of the Lot 1 larvae and 88% of the Lot 3 larvae. By Day 15, when no aneuploids or tetraploids were found, between 76% (Lot 1) and 88% (Lot 2) of the total population of larvae were triploid. By Day 54 the level of triploidy in all PB2 lots had remained high, with 84% to 91% of all larvae being triploid.

The growth rates of scallop larvae from Experiment 1 are illustrated in Figs. 3-5. Growth rates were poor in all treatments, with shell lengths increasing at rates of approximately 2 µm/day.

Table 2: Effects of blocking Polar Body 1 and Polar Body 2 on induction of triploidy in sea scallops, *Placopecten magellenicus*, in Experiment 1. Values are the percentage of larvae in the entire population that are triploid.

AGE (Days)	Lot #	Control	PB1 Block	PB2 Block
3	1	41	44	59
15	1	45	72	76
54	1	49	70	84
3	2	34	35	51
15	2	35	84	88
54	2	51	75	85
3	3	13	60	88
15	3	6	57	85
54	3	0	59	91

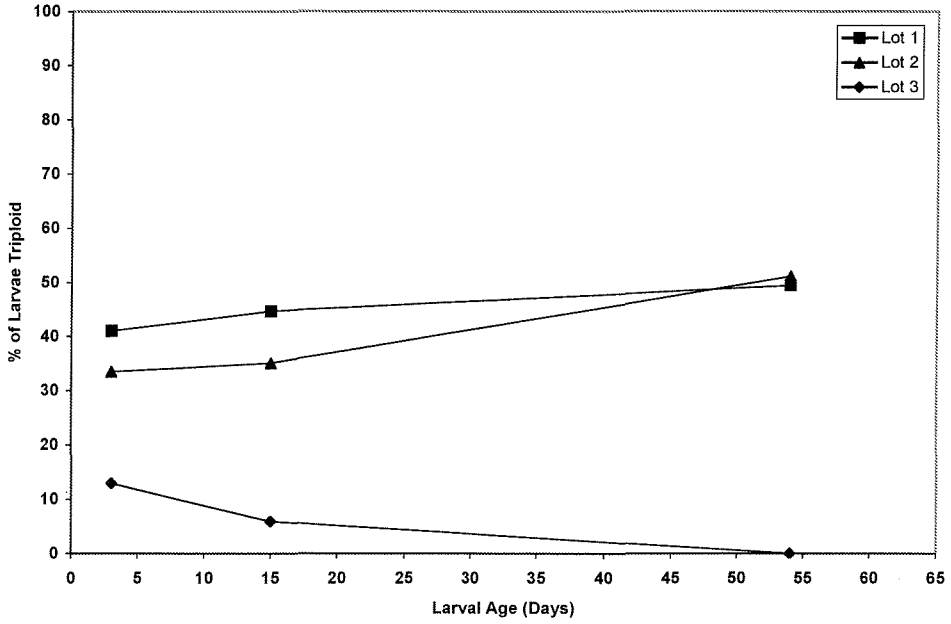

Fig. 1. Changes in the triploid composition of larval sea scallops, Control treatments, Experiment 1.

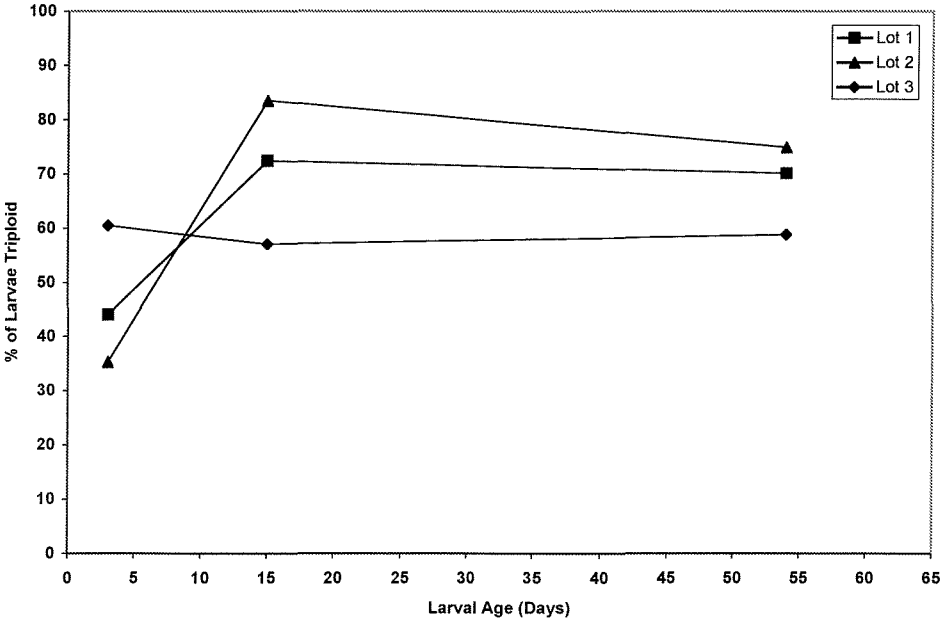

Fig. 2. Changes in the triploid composition of larval sea scallops, Polar Body 1 blocking treatments, Experiment 1.

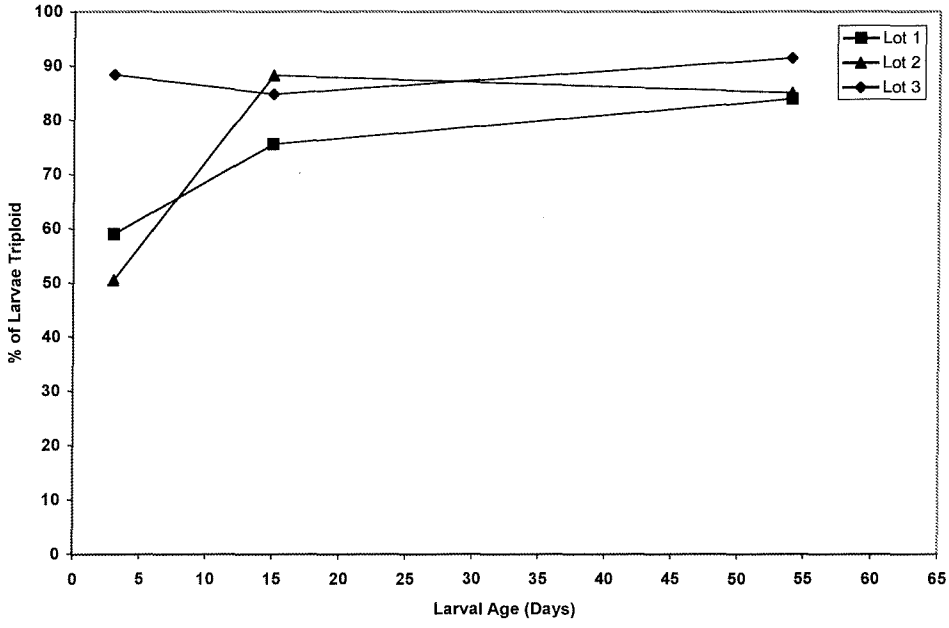

Fig. 3. Changes in the triploid composition of larval sea scallops, Polar Body 2 blocking treatments, Experiment 1.

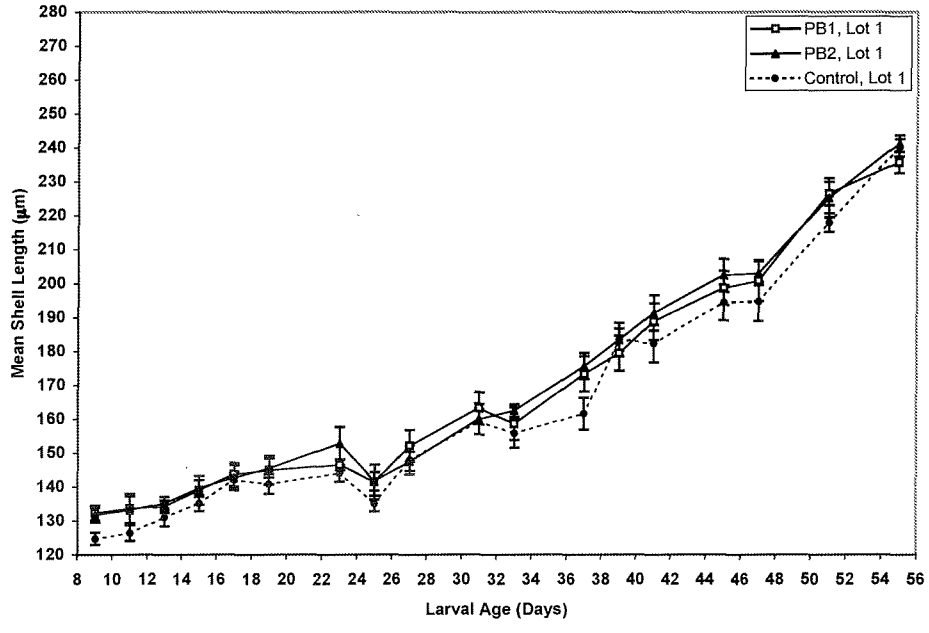

Fig. 4. Growth rates of larval sea scallops from Spawning Lot 1, Experiment 1. (Mean shell length ± standard error).

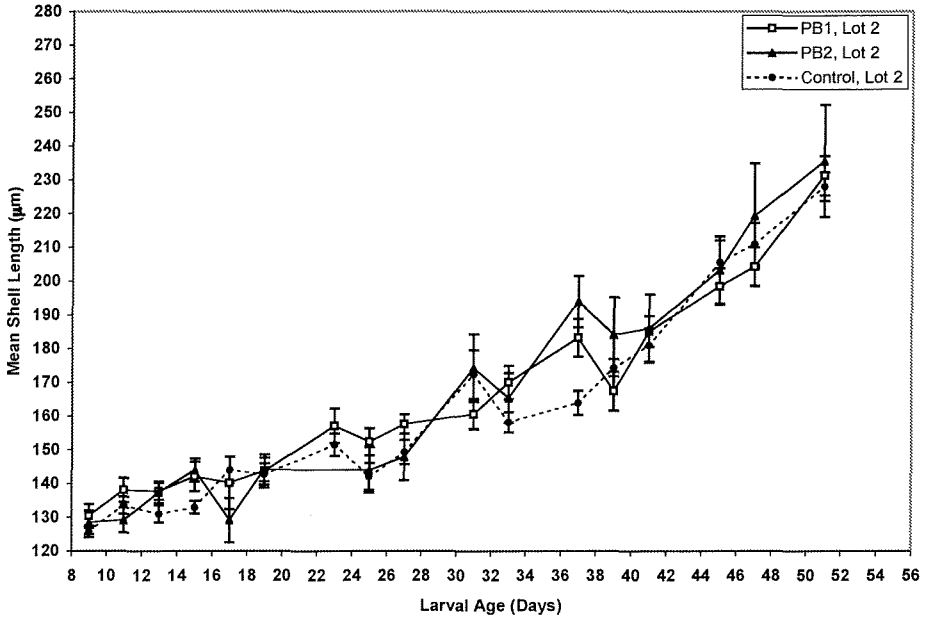

Fig. 5. Growth rates of larval sea scallops from Spawning Lot 2, Experiment 1. (Mean shell length ± standard error).

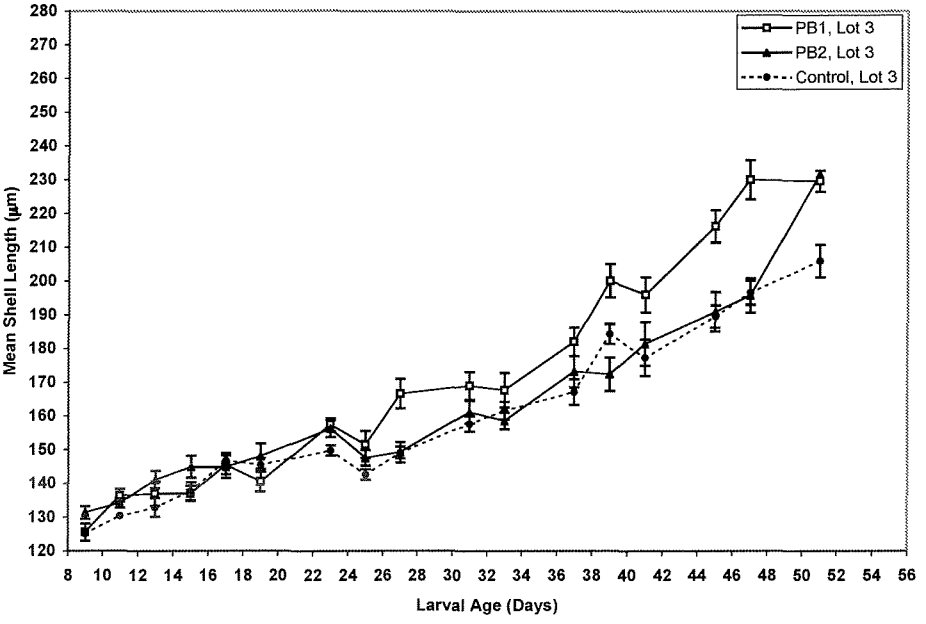

Fig. 6. Growth rates of larval sea scallops from Spawning Lot 3, Experiment 1. (Mean shell length ± standard error).

EXPERIMENT 2: INCUBATION TEMPERATURE AND CB CONCENTRATION TRIAL, SUMMER 2001

Table 3 and Figs. 7-10 summarize the results of the ploidy analyses from Experiment 2. Higher initial triploid induction percentages were found in the treatments that were incubated at 14°C; the highest triploid percentage found amongst the groups incubated at 6°C was only 25%. Within the 6°C treatments, the dosage of Cytochalasin B (0.50 mg/l vs. 0.25 mg/l) did not have much of an effect upon the initial level of triploidy (24% vs. 25% in Lot 1, and 16% vs. 13% in Lot 2). The concentration of CB did have more of an effect when embryos were incubated at 14°C, especially in Lot 2; 61% of the initial population were triploid in the 0.50 mg/l treatment, as opposed to 39% in the 0.25 mg/l group. As was also observed in Experiment 1, the proportion of triploids in all groups was higher on Day 11 than on Day 4, which can be attributed to the concomitant loss of tetraploids and aneuploids from the populations. In all treatments the proportions of triploids dropped off over time, but the two 14°C, 0.50mg/l groups maintained the highest numbers of triploids throughout the experiment (Figs. 9 and 10). Surviving competent pediveligers from all groups were transferred to downwellers for the juvenile grow-out phase, but growth rates were very low and all animals were dead within a year.

Growth rates were low in all treatments, ranging between 1.3 and 3.1 μm day^{-1} (see Figs. 11-14), and some of the lowest growth rates were in the 14°C incubation controls. Within incubation temperature treatments, growth rates were similar regardless of CB concentration, although amongst the Lot 2, 6°C incubation groups the larvae that were exposed to 0.25 mg/l CB grew at a faster rate than the 0.50 mg/l and the control treatment larvae after Day 30 (Fig. 13).

Table 3: Effects of using different incubation temperatures and concentrations of Cytochalasin B on induction of triploidy in sea scallops, *Placopecten magellenicus*, in Experiment 2. Values are the percentage of larvae in the entire population that are triploid.

AGE (Days)	Lot #	6°C 0.25 mg/l CB	6°C 0.50 mg/l CB	6°C Control	14°C 0.25 mg/l CB	14°C 0.50 mg/l CB	14°C Control
4	1	25	24	16	61	59	41
11	1	43	50	40	66	88	54
19	1	19	22	13	42	87	34
55	1	5	14	5	9	50	8
4	2	13	16	20	39	61	22
11	2	22	38	28	52	74	33
19	2	-	30	16	34	-	28
55	2	16	14	13	22	41	2

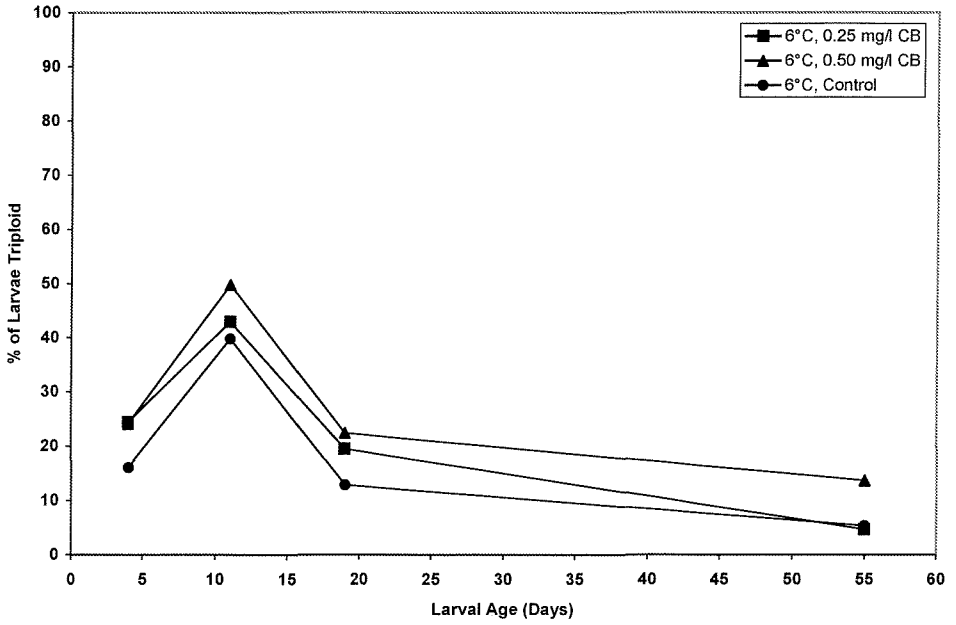

Fig. 7. Changes in the triploid composition of larval sea scallop populations, Spawning Lot 1, 6°C incubation, Experiment 2.

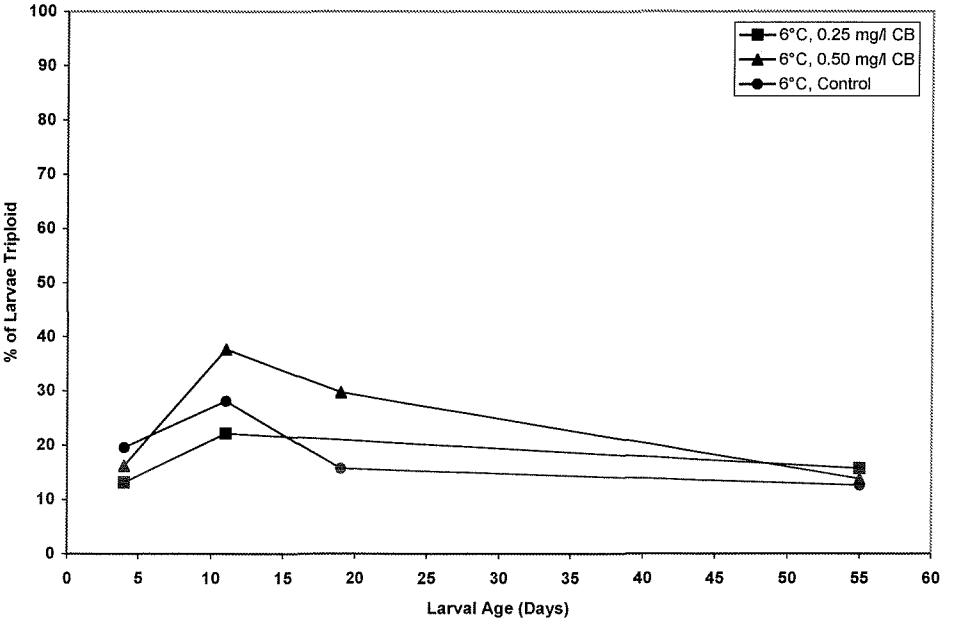

Fig. 8. Changes in the triploid composition of larval sea scallop populations, Spawning Lot 2, 6°C incubation, Experiment 2.

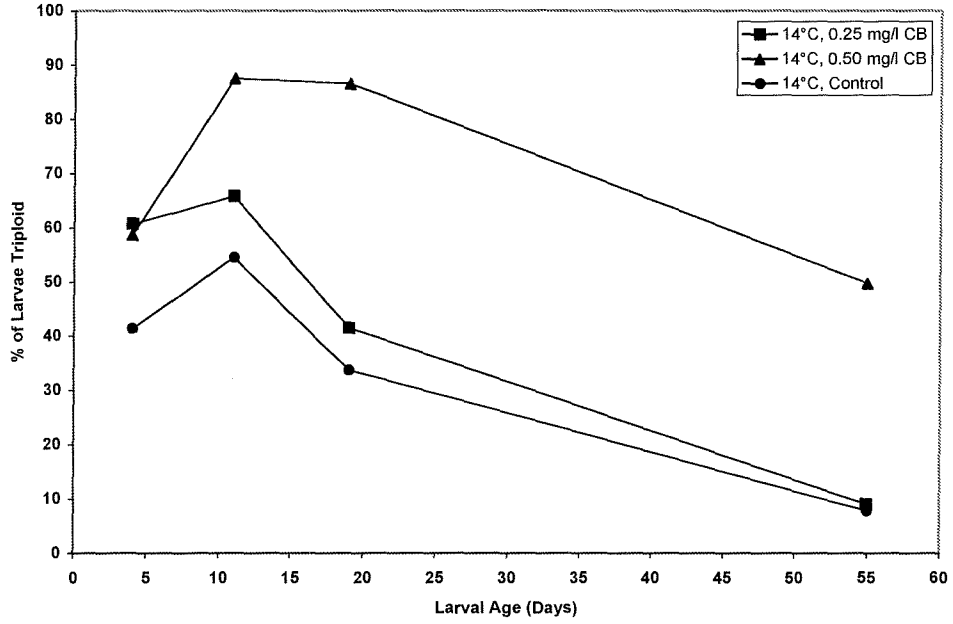

Fig. 9. Changes in the triploid composition of larval sea scallop populations, Spawning Lot 1, 14°C incubation, Experiment 2.

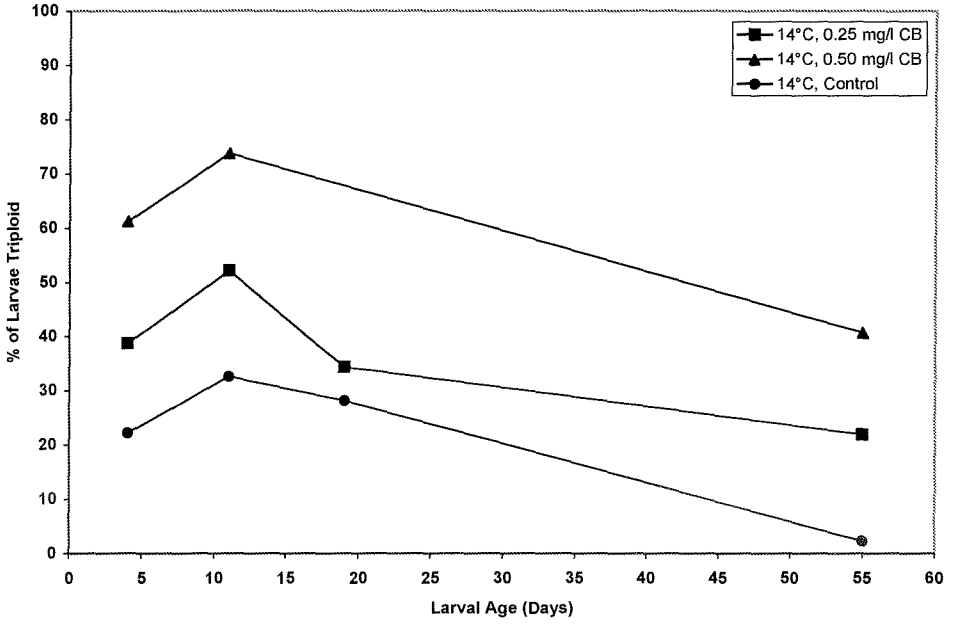

Fig. 10. Changes in the triploid composition of larval sea scallop populations, Spawning Lot 2, 14°C incubation, Experiment 2.

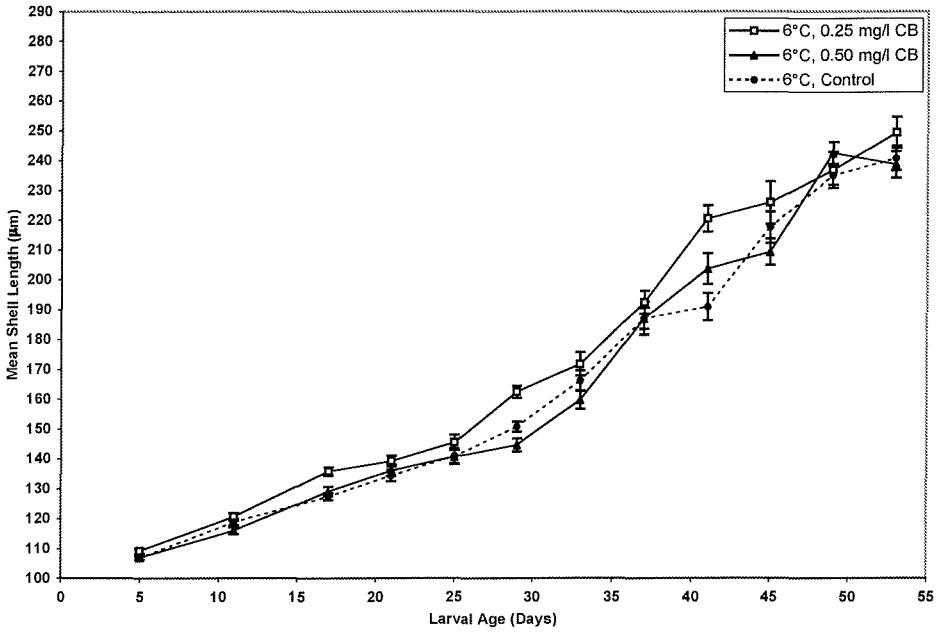

Fig. 11. Growth rates of larval sea scallops from Spawning Lot 1, 6°C incubation, Experiment 2. (Mean shell length ± standard error).

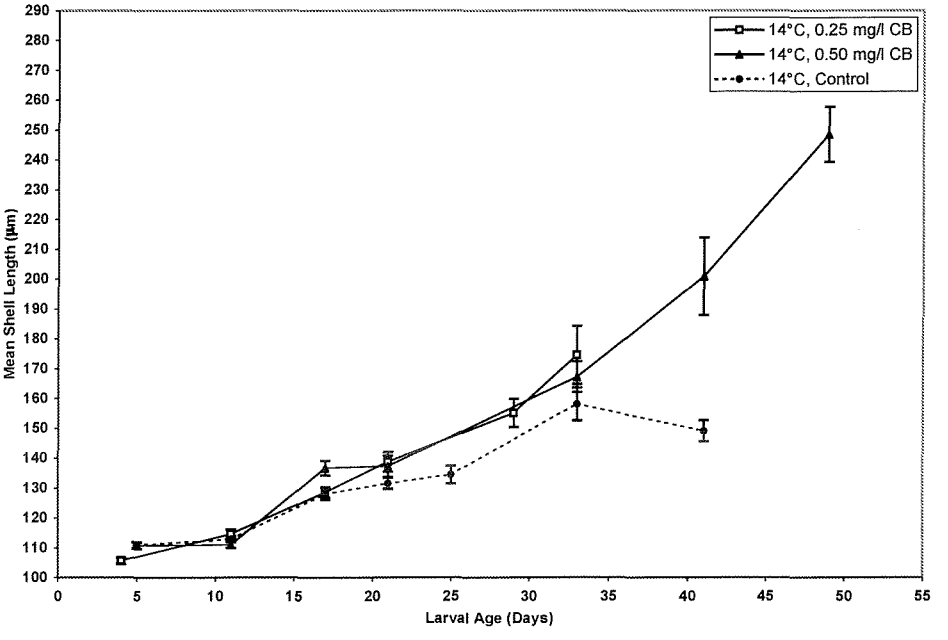

Fig. 12. Growth rates of larval sea scallops from Spawning Lot 1, 14°C incubation, Experiment 2. (Mean shell length ± standard error).

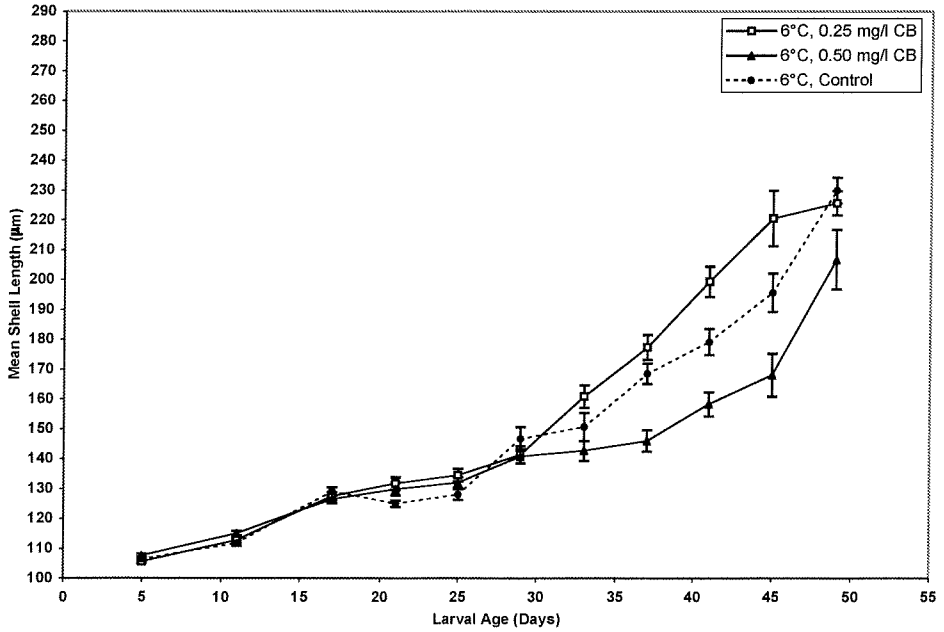

Fig. 13. Growth rates of larval sea scallops from Spawning Lot 2, 6°C incubation, Experiment 2. (Mean shell length ± standard error).

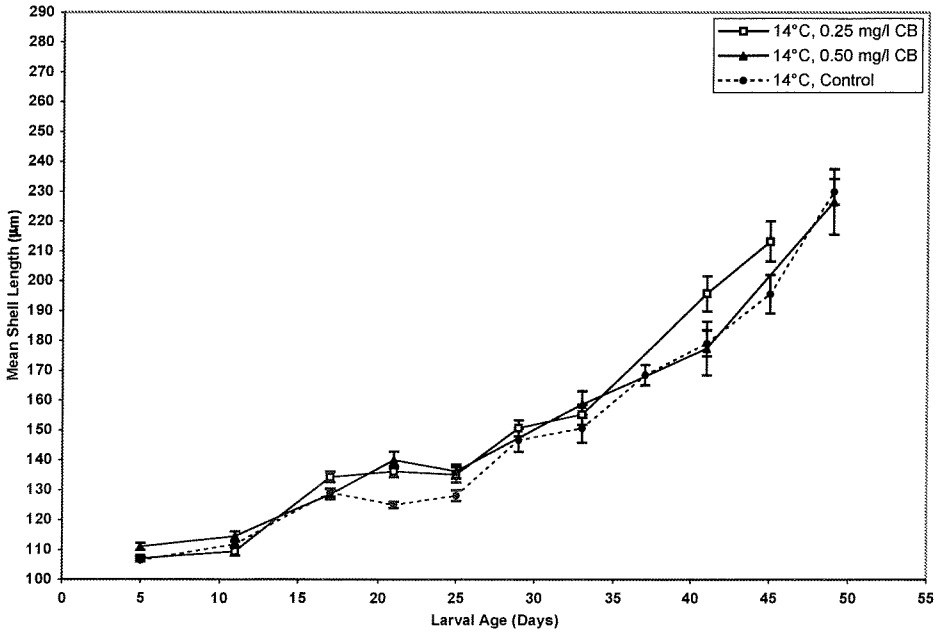

Fig. 14. Growth rates of larval sea scallops from Spawning Lot 2, 14°C incubation, Experiment 2. (Mean shell length ± standard error).

EXPERIMENT 3: "OPTIMUM INDUCTION CONDITIONS" TRIAL #1, FALL 2001

The triploidy induction methods used in Experiment 3 were very successful in producing a high amount of triploid larvae (see Table 2 and Fig. 15). On Day 3, 98% of all larvae in the group treated with 0.50 mg l^{-1} Cytochalasin B and incubated for 20 minutes at 14°C were triploid. This proportion stayed relatively constant throughout the duration of the larval period, with 94% of all larvae alive on Day 41 being triploid. No triploids were found in the control group. Scallop larvae from the CB-treated group grew at a faster rate (1.8 µm shell height per day) than the control larvae (1.0 µm/day) (Fig. 16).

Table 4: Effects of blocking Polar Body 2 with Cytochalasin B, 0.50 mg/l, at an incubation temperature of 14°C, on induction of triploidy in sea scallops, *Placopecten magellenicus*, in Experiment 3. Values are the percentage of larvae in the entire population that are triploid.

AGE (Days)	Control	CB-1
3	0	98
7	0	96
9	0	92
15	0	94
17	0	96
21	0	94
27	0	85
41	0	94

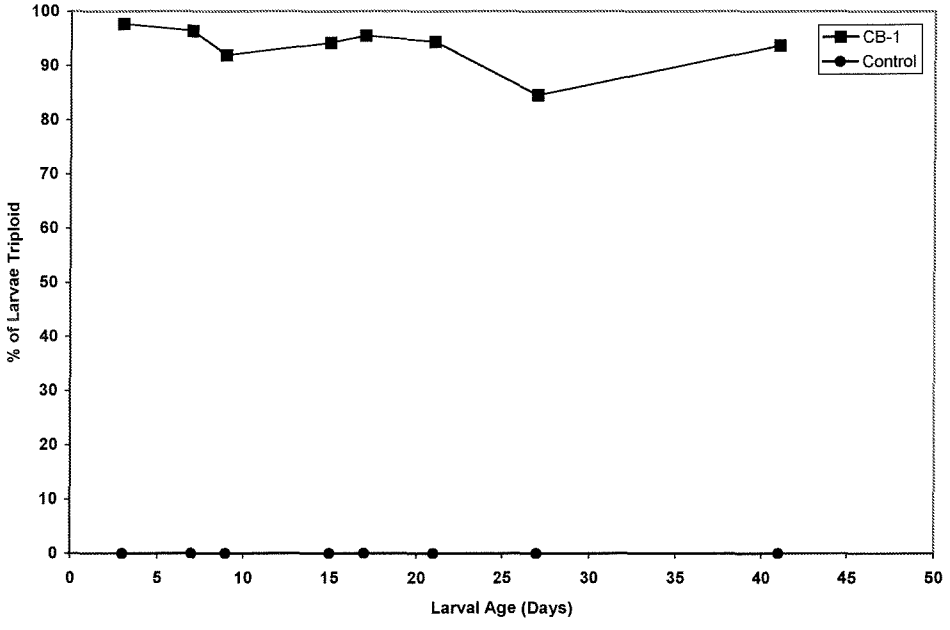

Fig. 15. Changes in the triploid composition of larval sea scallop populations, Experiment 3.

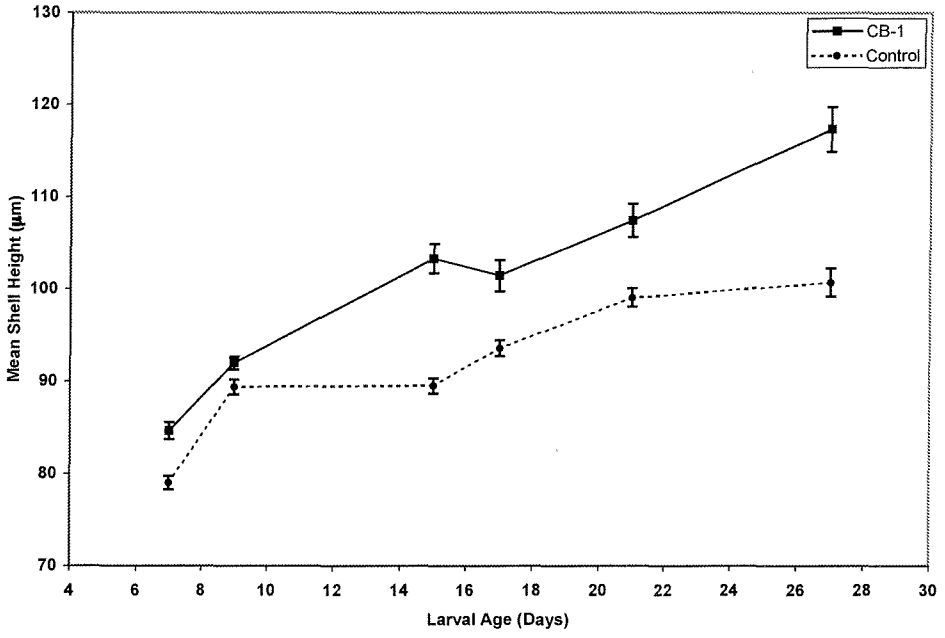

Fig. 16. Growth rates of larval sea scallops from Experiment 3. (Mean shell height ± standard error).

EXPERIMENT 4: "OPTIMUM INDUCTION CONDITIONS" TRIAL #2, SPRING 2002

The results from the ploidy analyses conducted during Experiment 4 are summarized in Table 5 and Fig. 17. Triploid induction was again successful, with 85% of the larvae in the first spawning lot and 95% in the second lot being triploid. These numbers decreased over the course of the experiment, dropping to 67% in the CB-1 group at 32 days of age. Larvae in the CB-2 group had all died by Day 20. The CB-MIX group, made up of surplus larvae from the CB-1 and CB-2 spawning lots that were pooled together, consisted of 61% triploids on Day 10, decreasing to 47% on Day 32. After being combined together on Day 38, the remaining larvae from the CB-MIX and CB-1 groups were comprised of 55% triploids on Day 50. No triploids were found in the control treatment. Rates of larval growth in the control group and the CB group were very similar (Fig. 18).

Table 5: Effects of blocking Polar Body 2 with Cytochalasin B, 0.50 mg/l, at an incubation temperature of 14°C, on induction of triploidy in sea scallops, *Placopecten magellenicus*, in Experiment 4. Values are the percentage of larvae in the entire population that are triploid.

AGE (Days)	Control	CB-1	CB-2	CB-MIX	CB-COMBINED
3	0	85	95	-	-
10	0	73	53	61	-
15	0	70	93	46	-
32	0	67	-	47	-
50	0	-	-	-	55

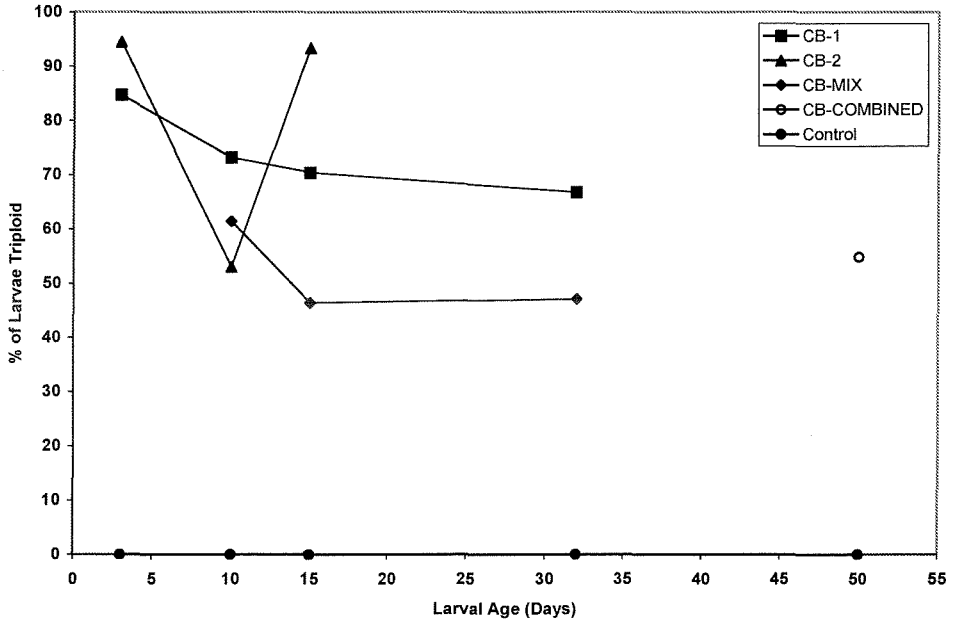

Fig. 17. Changes in the triploid composition of larval sea scallop populations, Experiment 4.

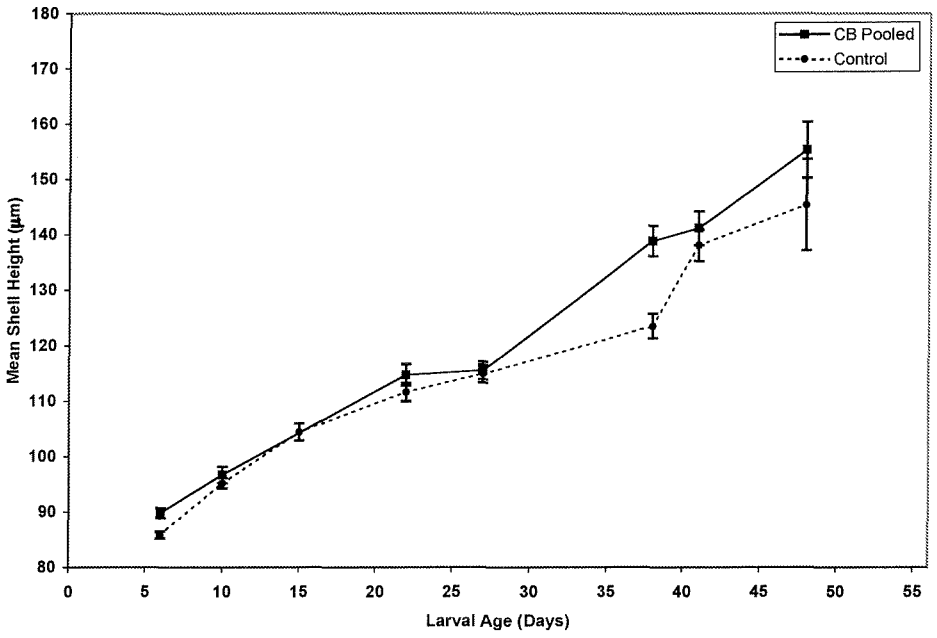

Fig. 18. Growth rates of larval sea scallops from Experiment 4. (Mean shell height ± standard error). CB-1 and CB-MIX group larvae were pooled together on Day 38.

EXPERIMENT 5: "OPTIMUM INDUCTION CONDITIONS" TRIAL #3, FALL 2002

The third trial to apply the optimum triploidy induction conditions (as defined by Experiment 2) was successful in producing large amounts of triploid sea scallop D-stage larvae (Table 6 and Figs. 19 and 20). Initial triploid induction percentages were 56% in the CB-1 group and 93% in the CB-2 treatment. These numbers did not change much in subsequent sampling up to Day 45 (Fig. 19). At 66 days of age only a few hundred larvae in each group were still alive, and these were transferred to downwellers for growout. The control treatment in this trial also contained some triploid larvae; 22% on Day 2, a percentage that remained fairly constant throughout the experiment. However, on Day 42 it was discovered that the control larvae were heavily infected with a protozoan, and all were terminated.

In the CB-3 and CB-4 groups, satisfactory ploidy analyses results were not obtained with the early samples, but at Day 14 the triploid percentages in those populations were 24% and 45% respectively. By Day 51 all control larvae were dead, and the numbers of triploids in the CB-3 and CB-4 groups had been reduced to 8% and 10%.

Larval growth in all CB groups did not differ significantly from their respective controls (Figs. 21 and 22).

Table 6: Effects of blocking Polar Body 2 with Cytochalasin B, 0.50 mg/l, at an incubation temperature of 14°C, on induction of triploidy in sea scallops, *Placopecten magellenicus*, in Experiment 5. Values are the percentage of larvae in the entire population that are triploid.

AGE (Days)	Control 1	CB-1	CB-2	Control 2	CB-3	CB-4
2	22	56	93	-	-	-
3	22	54	95	-	-	-
8	33	52	95	-	-	-
14	-	-	-	21	24	45
15	9	40	94	-	-	-
21	-	-	-	23	23	51
22	25	44	80	-	-	-
29	18	43	91	-	-	-
35	23	45	85	-	-	-
45	-	53	97	-	-	-
51	-	-	-	-	8	10

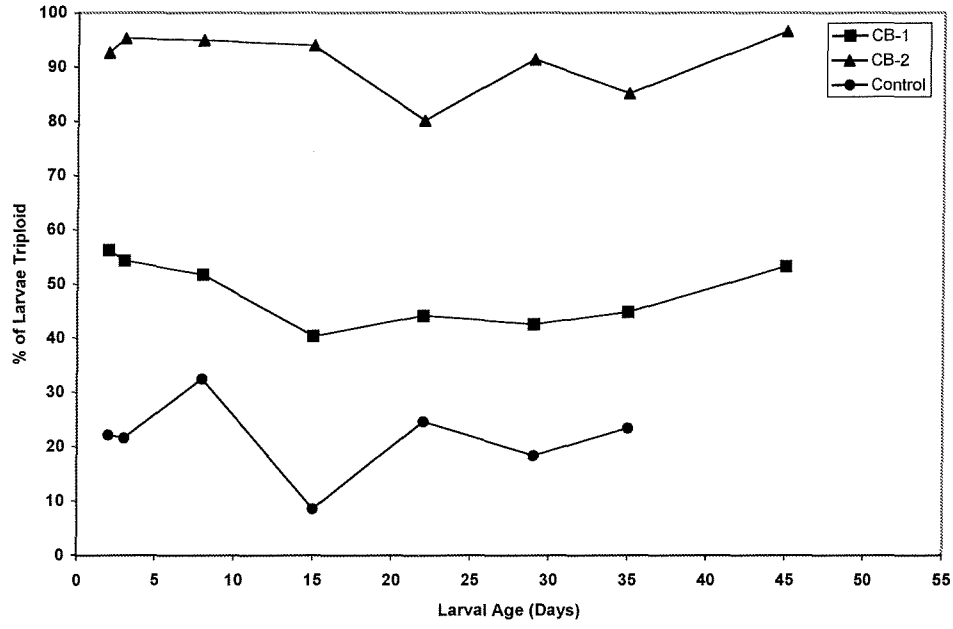

Fig. 19. Changes in the triploid composition of larval sea scallop populations, Experiment 5, CB-1 and CB-2 groups.

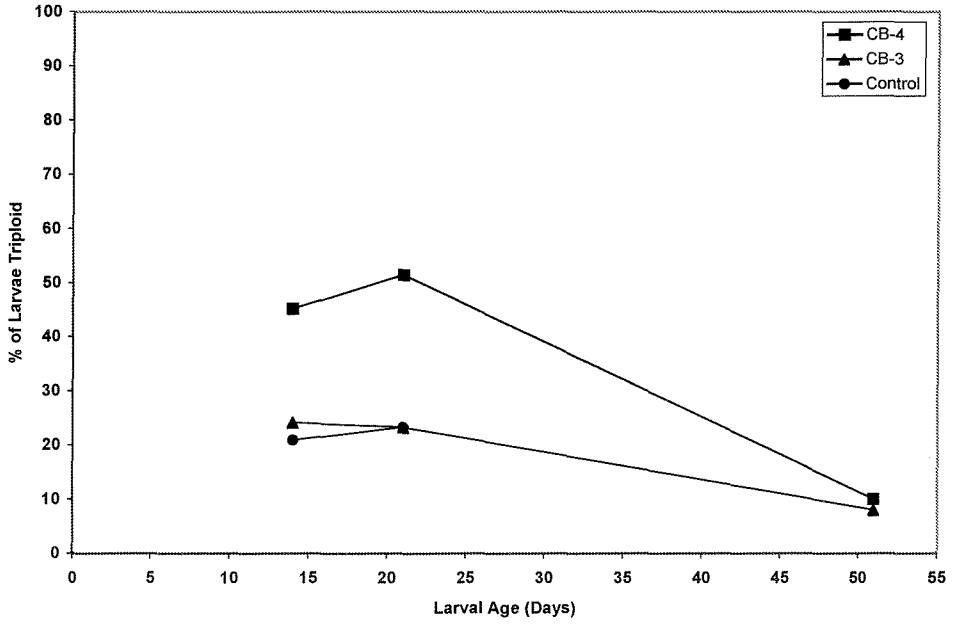

Fig. 20. Changes in the triploid composition of larval sea scallop populations, Experiment 5, CB-3 and CB-4 groups.

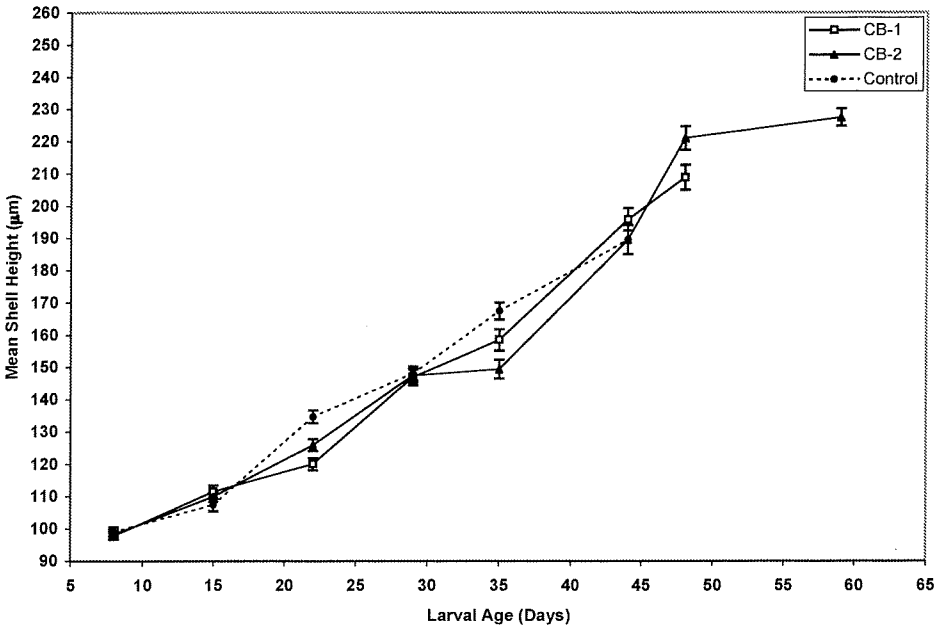

Fig. 21. Growth rates of larval sea scallops from Experiment 5, CB-1 and CB-2 groups. (Mean shell height ± standard error).

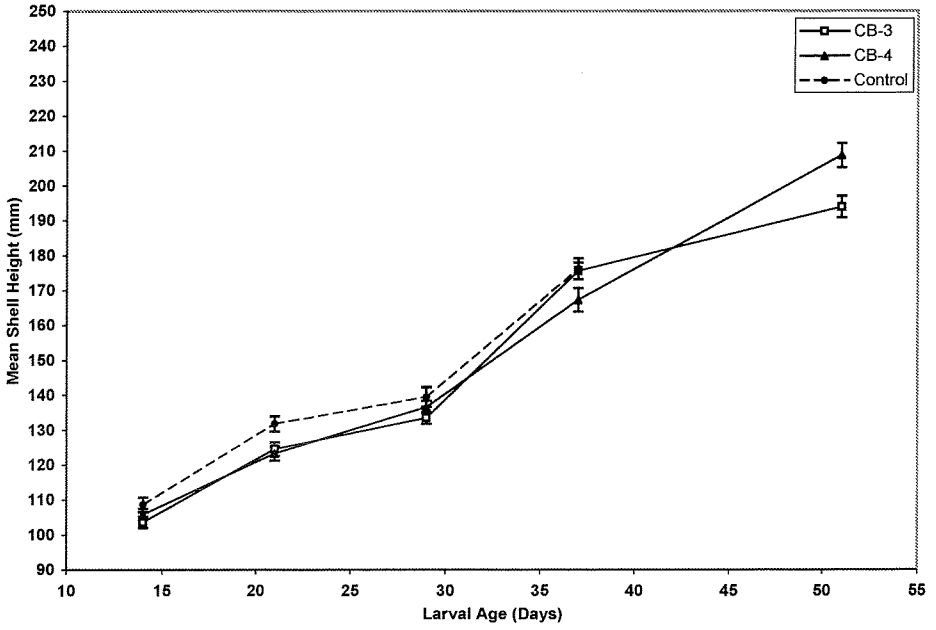

Fig. 22. Growth rates of larval sea scallops from Experiment 5, CB-3 and CB-4 groups. (Mean shell height ± standard error).

EXPERIMENT 6: HEAT SHOCK TRIAL #1, SUMMER 2001

The results of the ploidy analyses from Experiment 6 are summarised in Table 7 and Fig. 23. The initial effect of the heat shock treatment was moderately successful, in that 36% of larvae on Day 4 were found to be triploids. However, the proportion of triploids in the heat shock treatment declined to 23% on Day 11, and none were found on Day 19. Two percent of the larvae in the Day 55 sample were triploid. No triploids were found in the Control treatment on any date.

Table 7: Effects of blocking Polar Body 2 with heat shock on induction of triploidy in sea scallops, *Placopecten magellenicus*, in Experiment 6. Values are the percentage of larvae in the entire population that are triploid.

AGE (Days)	Control	Heat Shock Treatment
4	0	36
11	0	23
19	0	0
55	0	2

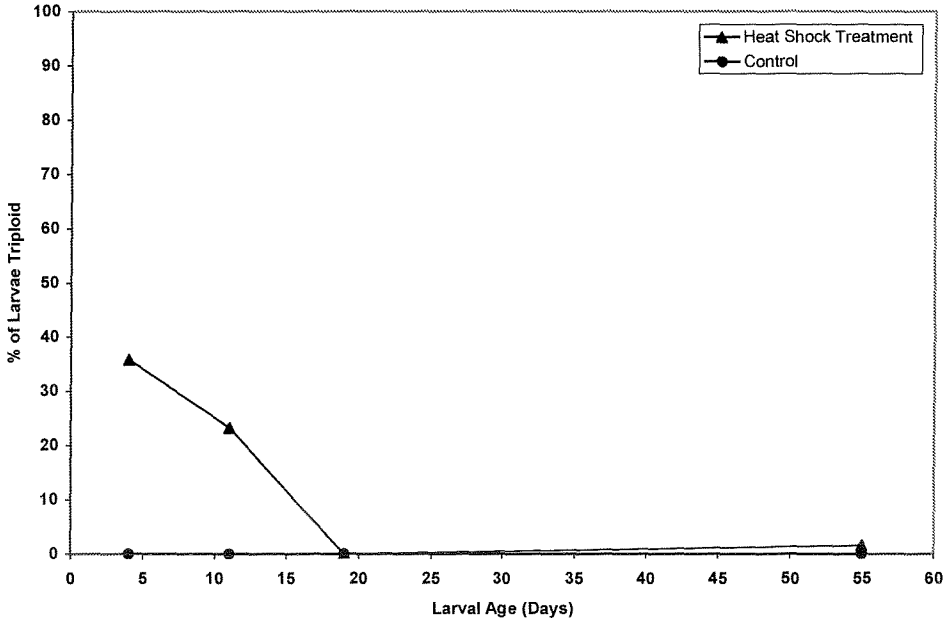

Fig. 23. Changes in the triploid composition of larval sea scallop populations, Experiment 6.

EXPERIMENT 7: HEAT SHOCK TRIAL #2, FALL 2001

Mixed results were obtained in the trials to study the effects of different levels of heat shock on triploid induction (see Table 8 and Fig. 24). No larvae survived the 30°C heat shock treatment, and when examined three days after fertilisation only a few embryos appeared to have developed to the 2-cell stage before death. In the two 25°C heat shock treatments, 26% and 31% of the larvae were found to be triploid on Day 3, although this proportion steadily declined until none were found in the samples on Day 9. No triploids were found in the Lot 1 20°C treatment, although 29% of the larvae in the second 20°C treatment were triploid on Day 3. By Day 7 this proportion had declined to only 1%, and none were found on Day 9, when the experiment was terminated. No triploids were found amongst the control larvae.

Table 8: Effects of blocking Polar Body 2 with different levels of heat shock on induction of triploidy in sea scallops, *Placopecten magellenicus*, in Experiment 7. Values are the percentage of larvae in the entire population that are triploid.

AGE (Days)	Lot #	20°C Heat Shock	25°C Heat Shock
3	1	0	26
7	1	0	21
9	1	0	0
3	2	29	31
7	2	1	23
9	2	0	0

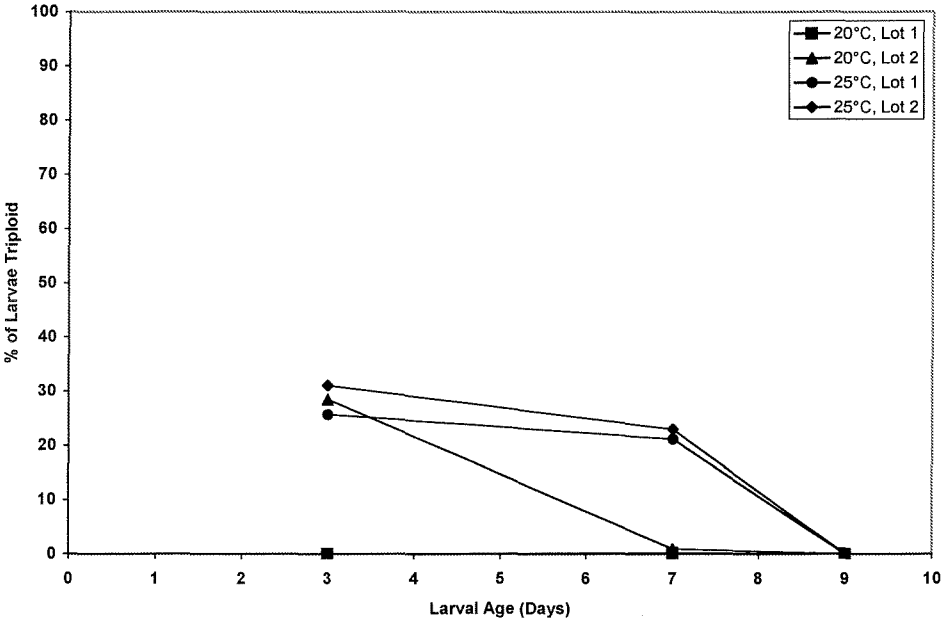

Fig. 24. Changes in the triploid composition of larval sea scallop populations, Experiment 7.

EXPERIMENT 8: 6-DMAP TRIAL #1, SPRING 2002

Rates of development were slow in the larvae treated with 6-DMAP; on Day 4, most animals were still in the gastrula or trochophore stages, as compared to the Control larvae which had developed to D-stage. Microscopic examination of larvae from the 6-DMAP treatment revealed that there was a high proportion of deformed animals, although some normal D-stage larvae were also present. After sampling for ploidy analyses, all groups of larvae were combined together in a single 20 litre bucket, since the numbers of viable larvae were so low. By Day 12, fewer than 10 larvae were still alive and the experiment was terminated.

The results of the ploidy analyses from Day 4 are summarized in Table 9. Of the larvae that had survived the PB2 blocking treatment, the lower dosage of 6-DMAP (200μM) resulted in 52% of all larvae in Lot 1 and 77% of larvae in Lot 2 being triploid. Greater levels of triploidy were found in the groups receiving the higher dose of 6-DMAP (400μM), with 60% in Lot 1 and 94% in Lot 2. No triploids were found in the control treatment sample.

Table 9: Effects of blocking Polar Body 2 with different levels of 6-DMAP on induction of triploidy in sea scallops, *Placopecten magellenicus*, in Experiment 8. Values are the percentage of larvae in the entire population that are triploid.

AGE (Days)	Lot #	200μM 6-DMAP	400μM 6-DMAP
4	1	52	60
4	2	77	94

EXPERIMENT 9: 6-DMAP TRIAL #2, FALL 2002

Results similar to those observed in Experiment 8 were found in the second trial with 6-DMAP. When examined at three days of age only a few D-stage larvae were seen, with most of the animals appearing to be quite deformed. The experiment was terminated at this time, after a sample was taken for ploidy analysis. Seventy percent of the larvae from this sample were triploid.

DISCUSSION

TRIPLOIDY INDUCTION TECHNIQUES

Meiosis I And Meiosis II Suppression; Experiment 1

Triploidy can be induced by suppressing either meiosis I (MI triploids) or meiosis II (MII triploids) by blocking polar body 1 or polar body 2 formation, respectively (Beaumont and Fairbrother, 1991). Blocking of polar body 1 has been shown to produce larval bivalve triploids that may grow faster than MII triploids (Guo *et al.*, 1992; Hawkins *et al.*, 1994), but this procedure often results in higher mortality and a greater proportion of aneuploids (Guo *et al.*, 1992; Nell, 2002). The first experiment of this study was designed in part to determine which of these two meiotic divisions should be targeted in order to produce the better yield of triploid sea scallop larvae, in addition to determining if Cytochalasin B (CB) is an effective triploidy induction agent in sea scallops.

As was the case with most bivalve species studied to date (Scarpa *et al.*, 1994; Nell, 2002), CB was found in this study to be an effective agent for inducing triploidy in sea scallops, with initial triploid proportions ranging from 35% to 88%. However, more triploids were found in the treatments where PB2 was blocked (between 51% and 88%) than in the PB1 treated groups (35% - 60%). Similar observations were noted with *Crassostrea gigas* by Gerard *et al.* (1999), one of the few studies to present results on production of MI triploids. These authors offer explanations for why MI triploid induction is difficult, including gamete quality and the synchronicity of larval development, the variability of which renders specific targeting of PB1 blocking difficult. In addition, since MI triploids have to develop through meiosis II there is likely to be a higher incidence of aneuploidy (Guo *et al.*, 1992), an observation also noted in the present study in which all aneuploids had disappeared by Day 15 of the experiment. These results lead to a conclusion that suppressing meiosis II is the preferred means for inducing triploidy in *Placopecten magellanicus*, in accordance with the approach used in studies with most other bivalve species (Nell, 2002).

Incubation Temperature And Cytochalasin B Concentration; Experiment 2

The optimal concentrations of CB and the ideal conditions for triploidy induction using Cytochalasin B have been determined for bivalves such as *Crassostrea virginica* (Barber *et al.*, 1992), *Crassostrea gigas* (Allen *et al.* 1989), and *Saccostrea commercialis* (Nell *et al.*, 1996). Downing and Allen (1987), studying triploidy induction using CB in *Crassostrea gigas*, noted that the effectiveness of CB was dependent upon the temperature at which exposure to the chemical occurred. Many factors play a role in the synchrony of meiosis and therefore the effectiveness of CB exposure, including temperature dependent rates of the binding and release of CB to the appropriate receptors. In addition, complex relationships between temperature and rates of embryo development exist, especially at elevated temperatures where microtubules are inhibited

thereby arresting chromosome movement and making the embryos more susceptible to the action of CB (Downing and Allen, 1987). With *Crassostrea gigas*, the highest percentage of triploids was found in treatments where CB was applied for 15 minutes at 25°C (Downing and Allen, 1987), a temperature that is 5°C above normal rearing conditions for these animals. For these trials with inducing triploidy in *Placopecten magellanicus*, it was decided to choose a similar temperature differential for studying the interdependence of CB concentration and temperature; 6°C is close to the normal rearing temperature for sea scallop broodstock in our lab, and 14°C was chosen as a suitably higher temperature that is within the physiological tolerance of sea scallops, but represents a considerable thermal increase over the ambient conditions. It is also common practice in most sea scallop hatcheries to rear larvae at 14°C.

In the current study, the highest percentage of triploids were obtained when embryos were incubated at 14°C in a CB concentration of 0.50 mg/l for 20 minutes. At an incubation temperature of 14°C, the concentration of CB played a significant role in the success of triploidy induction; much higher yields were obtained in the 0.50 mg/l CB treatments than in those treated with a CB concentration of 0.25 mg/l. This effect was not so pronounced at the lower incubation temperature (6°C), where similar results were obtained regardless of CB concentration. These results seem to coincide with those reported by Downing and Allen (1987), and the likely explanation is the influence of temperature on rates of CB binding and release, and on the action of the cytoskeletal microtubules during meiosis II. As development is directly influenced by temperature, lower temperatures would have slowed down chromosome movement, thereby extending meiosis beyond the induction window. Applying this model to a cold water species like *Placopecten magellanicus*, increasing the temperature to a level at the higher end of its normal range of thermal tolerance during CB exposure would increase the activity of Cytochalasin B while simultaneously disrupting chromosome migration during polar body formation, leaving embryos more susceptible to the actions of CB over a longer window of vulnerability (Downing and Allen, 1987).

Optimum Conditions Trials, Experiments 3, 4, and 5

After defining the optimum conditions for inducing MII triploidy in sea scallops in Experiment 2, Experiments 3, 4, and 5 were all conducted with the goal of producing large amounts of triploid larvae. The intended purpose of these larvae was twofold; one, to rear the animals in lab culture and assess the performance of triploid sea scallop juveniles and adults, and secondly, to attempt to produce tetraploid sea scallops for use as broodstock.

The potential offered by the use of tetraploids in bivalve culture has received a considerable amount of attention in recent years (Guo *et al.*, 1996a; Eudeline *et al.*, 2000a,b; Nell, 2002). Their benefits stem from the fact that tetraploids have normal fertile gonads and are capable of producing diploid gametes. When crossed with normal diploids, the offspring are 100% triploids that

are completely sterile and never revert back to diploids (Guo *et al.*, 1996). This presents the aquaculture industry with great advantages, since chemical and physical methods of inducing triploidy are rarely 100% effective, and the elimination of chemicals altogether reduces costs and avoids any toxicity-related concerns of the consumer.

To date, the only successful method for producing tetraploids has been according to the procedures developed by Guo and Allen (1994b). In this approach, eggs from triploid oysters that have partially developed gonads are fertilized with sperm from diploids, followed by inhibition of polar body 1 formation. Most of the progeny develop as aneuploids, but a small number are tetraploid and develop normally to maturity (Guo and Allen, 1994b). Tetraploid broodstock oysters were then successfully used to produce all triploid offspring when mated with diploids (Guo *et al.*, 1996a), and these larvae grew at higher rates than triploids produced via CB blocking of meiosis II (Wang *et al.*, 1999). The process has since been applied successfully to produce tetraploid *Crassostrea virginica* (Supan, 2000).

The success achieved with other bivalve species led to this study's interest in producing tetraploid *Placopecten magellanicus* as well. Unfortunately, the production of adult triploids was not achieved in these experiments (see discussion of larval performance below), and these results precluded any attempts to produce tetraploid sea scallops. However, the CB treatment protocols as defined in Experiment 2 and refined throughout Experiments 3, 4, and 5 proved effective in producing large numbers of triploid larvae, with initial proportions of triploids in some treatments being as high as 98% (Experiment 3) and 95% (Experiment 4, CB-2 group). In some other treatments, however, initial triploidy induction success was not as high, notably in Experiment 5, groups CB-1, CB-3, and CB-4. This may be the result of poorer gamete quality (Gerard *et al.*, 1999), or mismatching of the application of the CB treatment with the critical window for polar body blocking due to asynchronous meiotic development (Allen *et al.*, 1989).

Heat Shock As A Triploidy Induction Agent; Experiments 6 and 7

Temperature shock has also been used as a mechanism for inducing triploidy in bivalves, including *Crassostrea gigas* (Quillet and Panelay, 1986; Yamamoto *et al.* 1988), *Mytilus edulis* (Beaumont and Kelly, 1989), *Mytilus galloprovincialis* (Scarpa *et al.*, 1994), and *Mytilus chilensis* (Toro and Sastre, 1995). Mixed results were reported by these authors, with some reports recording initial triploid percentages as high as 97% with *Crassostrea gigas* (Yamamoto *et al.* 1988), but others reporting not as much success, namely Toro and Sastre (1995), who found that only between 15% and 51% of the early larvae in their trials with *Mytilus chilensis* were triploid. Most of these authors have also reported that larval survival is often adversely affected by heat shock treatments.

In this study, heat shock applied during polar body 2 formation did not produce significant amounts of triploid larvae, and those triploids that were present in the populations did not survive for very long. In Experiment 6, a heat

shock of 22°C (8°C above ambient) resulted in only about 36% of the larvae at D-stage being triploid, and these had disappeared by Day 19. In Experiment 7 where 3 different levels of heat shock were applied, the highest temperature used (30°C) killed all larvae outright, while the two 25°C treatments resulted in triploidy percentages of only 26% and 31%. Interestingly, only one of the 20°C treatments produced any triploids at all (29%); none were found in the other replicate. This result may be indicative of missing the critical window for polar body blocking; timing of heat shock application is critical since eggs are vulnerable to physical perturbations for only a very short period of time (Allen, 1987; Beaumont and Fairbrother, 1991). For these reasons, among others, Cytochalasin B has usually been reported as being more effective than heat shock for inducing triploidy (Scarpa *et al.*, 1994; Nell, 2002).

6-Dimethylaminopurine As An Induction Agent; Experiments 8 and 9

6-Dimethylaminupurine (6-DMAP) has been used to induce triploidy in some bivalves, including *Crassostrea gigas* (Gerard *et al.*, 1999), *Saccostrea commercialis* (Nell *et al.*, 1996), and *Mytilus edulis* and *Placopecten magellanicus* (Desrosiers *et al.*, 1993). The potential advantages of using 6-DMAP over Cytochalasin B are based upon the toxic nature of CB, which is a suspected carcinogen that is not only hazardous to the lab personnel who handle it, but has also raised health concerns in the food industry over its use (Guo *et al.*, 1994). In addition, CB must be dissolved in the solvent dimethylsulfoxide (DMSO), which is in itself toxic and its use necessitates the addition of an additional rinsing step in the triploidy induction procedure (Desrosiers *et al.* 1993).

The use of 6-DMAP has been met with varying degrees of success. 6-DMAP was used to induce triploidy in the Sydney rock oyster *Saccostrea commercialis* (Nell *et al.*, 1996), however CB was found to be more effective with that species in terms of both triploidy induction and subsequent survival. Gerard *et al.* (1999) reported that some of their experiments using 6-DMAP with *Crassostrea gigas* resulted in triploid yields of up to 99%, and average yields of 84%. Desrosiers *et al.* (1993) studied the use of 6-DMAP as a triploid induction agent in the Pacific oyster, *Crassostrea gigas*, the sea scallop, *Placopecten magellanicus*, and the blue mussel, *Mytilus edulis*. It was found to be effective in sea scallops when administered 70 minutes after fertilization at a dosage of 400 µM 6-DMAP for 15 minutes, producing up to 95% triploids.

In an attempt to replicate the success of Desrosiers *et al.* (1993), the conditions defined by those authors were used as a guideline for the present study. Experiment 8 used two concentrations of 6-DMAP (200 µM and 400µM) for an exposure duration of 20 minutes, conditions which proved optimal for triploidy induction for Desrosiers *et al.* (1993). The results from Experiment 8 differed greatly from those reported in the earlier study. Larval development was slower in the 6-DMAP treatments, there was a much higher incidence of deformed larvae than was seen in control groups, and survival to D-stage was very low. Initial triploid yield was 52% and 77% in the 2 replicates at the lower dosage and 60%

and 94% in the higher dosage treatments. These latter numbers compare favourably with those reported by Desrosiers *et al.* (1993), but those authors did not report the higher mortality and abnormalities that were found in this study. In Experiment 9, using a 6-DMAP dosage of 400 µM for only 15 minutes, mortality was very high and most larvae had developed abnormally before death. Of the very few animals that had survived to D-stage however, approximately 70% were triploid. Both Desrosiers *et al.* (1993) and Zhang *et al.* (1998; working with Pacific abalone) reported that longer incubation durations with 6-DMAP and higher dosages of the chemical resulted in more abnormally developed larvae. Zhang *et al.* (1998) state that if the length of 6-DMAP exposure extends throughout the entire duration of meiosis II and into the first mitotic division, then survival and normal development are seriously affected. It is likely that this mechanism explains the poor results found in this study, even though similar conditions were used in all experiments. Variability in developmental synchrony and differences in duration of meiosis II may be an explanation for the different results reported in this study.

PERFORMANCE OF TRIPLOID SEA SCALLOP LARVAE

Survival

As has been documented with several bivalve species, the use of Cytochalasin B to induce triploidy results in lower survival rates to veliger stage as compared to controls (e.g., *Crassostrea virginica* (Stanley *et al*, 1981); *Tapes philippinarum* (Dufy and Diter, 1990); *Crassostrea gigas* (Downing and Allen, 1987; Beaumont and Fairbrother, 1991); *Argopecten ventricosus* (Ruiz-Verdugo *et al.*, 2001). Cytochalasin B-related toxicity can be responsible for between 50% and 99% mortality in some triploid induction treatments (Allen *et al.*, 1989). Downing and Allen (1987) also report that the mortality arising from CB use is exacerbated when CB is applied at certain critical stages of development, including recently fertilized embryos and during polar body 1 formation. Toro and Sastre (1995) suggest that much of this mortality arises as a function of increased early mortality amongst the high proportion of aneuploids that are commonly found in CB-induced triploid embryos. In the current study with *Placopecten magellanicus*, precise calculations of survival rates from fertilization to D-stage were not made, but it was generally noted that the numbers of larvae reaching D-stage in the CB-treated groups were much lower than those found in the corresponding controls. These observations are similar to results reported in other bivalve species by the aforementioned authors.

For most triploid bivalves, survival rates from D-stage to metamorphosis are similar in control and triploid groups (Allen, 1987; Downing and Allen, 1987; Beaumont and Fairbrother, 1991; Nell *et al.*, 1995). However, Desrosiers *et al.* (1993) noted that survival rates in *Crassostrea gigas* larvae treated with CB and 6-DMAP were similar to each other but lower than those seen in the control treatments, leading those authors to conclude that triploid larvae may be less hardy than diploids. In the present study, smaller larvae were routinely culled out

of the populations as a means of keeping the larval cultures healthy, so data on comparative survival rates were not collected. However, no obvious differences in survival rates were noted in any of these experiments; survival to metamorphosis was very low regardless of treatment.

The production of adult triploids was not achieved in the current study; in all experiments, it proved difficult to rear sea scallop larvae through settlement. This situation held true for animals in the control treatments as well as the putative triploids from all 3n induction experiments. Low larval survival rates largely appeared to be closely related to the poor growth observed in larvae from this study. Growth rates were low in all groups, and the accumulated natural mortality resulting from an extended larval period (Rumrill, 1990) left very few individuals alive by the time they were ready to metamorphose, a physiologically stressful event even under the best of conditions. Larvae in all experiments were well supplied with adequate levels of food and showed evidence of feeding as seen by microscopic examination of stomach contents and of the lipid content in the digestive gland (Jackson, 1993), but this apparent nutritional intake was not reflected as substantial growth rates. The reasons for the poor growth rates in these experiments are unknown, but may be related to problems with seawater quality.

Reversions

An interesting observation in some of the trials in this study was a steady rate of decline in the proportions of triploids in the populations. This may have been caused by one or both of two factors: reversion, or differential rates of mortality in the diploid and triploid populations. Reversions from triploid status to diploidy or to triploid/diploid mosaics (heteroploids) occasionally occur amongst triploid bivalves (Allen *et al.*, 1996, 1999; Shatkin *et al.*, 1997; Beaumont, 2000; Nell, 2002). Allen *et al.* (1996) suggest a possible model for this phenomenon, in which reversion occurs as a consequence of a disruption of spindle formation during mitoses of triploid cells. It has been reported that reversions may be responsible for reductions in the triploidy levels of Pacific oysters of up to 20% (Allen *et al.*, 1996).

Heteroploid reversion may be responsible for the declining proportions of triploids observed in some of the experiments in the current study, but it is difficult to distinguish reversion from differential rates of mortality in the diploid and triploid populations. For instance, Nell (2002) noted that in over a decade of work with triploid Sydney rock oysters, the proportions of triploids in cultures from the larval stage through to market size always increased, an observation which was believed to reflect better survival of the triploids. This author also noted, however, that any reversion that may have occurred in these animals would have been masked by the higher survivability of the triploids. A comparable situation was seen in Experiment 3 from this study, where approximately 98% of the larvae at Day 3 were triploid and the proportion of triploids in the population remained very high throughout larval development. Again, this result may indicate that triploid

and diploid mortality rates were similar throughout the larval period, but if reversions were occurring then their effect may have been countered by a higher rate of diploid mortality.

Growth

Improved growth rates are one of the principal reasons behind the interest in producing triploid bivalves, but it has been commonly reported that improved growth is not manifested during the larval stages of many species, including *Crassostrea gigas* (Downing and Allen, 1987; Desrosiers *et al.*, 1993), *Chlamys varia* (Baron *et al.*, 1989), *Tapes philippinarum* (Laing and Utting, 1994), and *Tapes dorsatus* (Nell *et al.*, 1995). On the other hand, some studies report that the triploid larvae of certain bivalves do indeed grow at faster rates than their diploid siblings (Wang *et al.*, 2002). For instance, Yamamoto *et al.* (1988) found that triploid meiosis I *Crassostrea gigas* larvae had significantly longer mean shell lengths than either meiosis II triploids or control larvae. Since meiosis I triploids theoretically have a higher level of heterozygosity than meiosis II triploids (Stanley *et al.*, 1984), and triploids in general are theoretically more heterozygous than diploids (Allendorf and Leary, 1984), these authors attributed their results to the phenomenon of "hybrid vigor," where increased growth rates are correlated with higher overall heterozygosity. However, heterozygosity alone probably cannot account for increased growth rates in triploid bivalves (Guo and Allen 1994a).

A comprehensive interpretation of the seemingly contradictory results reported in triploid and diploid larval growth rates requires a better understanding of the possible mechanisms behind the phenomenon of increased body size in triploids. The presumption that meiosis I triploids are more heterozygous than meiosis II triploids has been challenged by Guo and Allen (1994a), who note that this phenomenon is only applicable in situations where the recombination frequency is below 0.67 (Guo *et al.*, 1992). In addition, Mason *et al.* (1988) report that even though triploid *Mya arenaria* were found to be almost twice as heterozygous as diploids, they could not find any correlations between heterozygosity and growth rate. And of course, the energy budget of a bivalve larva does not include a quotient for reproductive effort, so there must be another mechanism at work that can account for the larger sizes seen in triploid bivalves.

Guo and Allen (1994a) presented a third hypothesis that describes polyploid gigantism as a mechanism to account for higher growth rates in triploid bivalves. Since triploid cells contain 50% more DNA than diploid cells, a correspondingly greater amount of cytoplasm may be necessary in order to support the larger nucleus and maintain an optimal ratio of cytoplasm to DNA, resulting in larger triploid cells. In molluscs the mosaic pattern of development controls and programs the number of cell divisions, unlike the regulative pattern of development seen in higher animals where increases in cell size are countered by a decrease in the total number of cells (Wang *et al.*, 2002). Thus, in molluscs the result is a triploid animal that has the same number of cells as a diploid, but since each of those cells is bigger the animal itself is correspondingly larger. It is likely

that all three proposed mechanisms contribute to producing larger triploids, but for larval bivalves any observed differences in growth rate between diploids and triploids reported in the literature are probably a reflection of polyploid gigantism acting in concert with hybrid vigor.

Many previous studies may not have revealed the impact of polyploidy gigantism on triploid growth rates because the expression of gigantism can be affected by environmental factors such as food availability (Guo and Allen, 1994a). According to this view, the cells of triploid animals require more nutritive input for maintenance and growth since they are larger, and in a nutritionally stressed situation triploid larvae may therefore not grow at rates greater than those of diploids. This hypothesis was expanded upon by Wang *et al.* (2002), who proposed a single unified theory that incorporates all three hypotheses explaining why triploid bivalves are bigger than diploids. In this view the larger cells arising from polyploid gigantism are primarily responsible, giving triploids the potential to be larger than diploids. Polyploid gigantism can thus be expressed in different ways, being magnified by higher heterozygosity or better nutritive input, or not expressed to the same degree if the animals are nutritionally stressed or less heterozygous.

Although there was no evidence to suggest that the scallop larvae in this study were underfed, larval growth rates were poor in all experiments. It is therefore likely that any potential growth advantages arising as a result of polyploid gigantism were likely masked by whatever environmental factors may have been responsible for the poor growth rates. In addition, most experiments did not reveal any substantial differences in larval growth rates between putative triploids and diploids. However, significant differences were seen in the two treatments of Experiment 3, the only trial of this study in which the treated group had a very high proportion of triploids (>90%) while the control group had none, rendering distinctions in larval growth rate as a function of ploidy more readily discernible. Since the putative triploids grew at a faster rate than the diploid controls, these data suggest that the cells of larval triploid *Placopecten magellanicus* may be larger than those of their diploid siblings, thereby supporting the polyploid gigantism hypothesis of Guo and Allen (1994a). On the other hand, the triploid larvae may have been more heterozygous than the diploids; whatever the reason, this is the first study to show that triploid *Placopecten magellanicus* larvae can exhibit faster growth rates than diploids. More research is clearly necessary in order to properly assess the relative contributions of polyploid gigantism, heterozygosity, and reproductive energy reallocation to the greater performance of triploid scallops and other bivalves (Nell, 2002).

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APPENDICES

1. General triploidy induction procedures
2. Instructions for making Propidium iodide nucleus staining solution
3. Detailed protocols for preparing samples for ploidy analysis via flow cytometry
    a. Larvae
    b. Juveniles and adults
4. Sample page output from ModFit ploidy analysis software

Appendix 1. General triploidy induction procedures for sea scallops

As a result of the trials conducted to date, the optimum procedures for conducting triploid induction with CB were well refined, and are reproduced in itemised form below.

Materials:

5 litre buckets (one for each spawning adult scallop)
10 litre buckets (to fertilize eggs in, and to use as the Cytochalasin B bath; one for each batch of embryos)
10 litre buckets (for DMSO bath; one for each batch of embryos)
10 or 5 litre buckets (for rinsing embryos into after DMSO bath and FSW rinse)
20 µm Nitex screens, large diameter (for rinsing Cytochalasin B and DMSO off embryos)
Mixing plungers, to mix embryos during fertilization, and during CB & DMSO baths.
Cytochalasin B (CB), pre-mixed in vials (1ml, at a concentration of 0.1 mg/ml in DMSO)
DMSO, pre-dispensed in vials (0.2 ml, to be used in 2 litres of seawater)
Multiwell culture plate, to monitor Polar Body (PB1) formation
Counter, for counting numbers of embryos that release PB1
Microscope, for observing polar body formation

Methods:

Cytochalasin B Treatments:
(In this case, block PB-2 with Cytochalasin B, 0.50mg/l, 14°C Incubation)
Replicate spawning lots/fertilizations/batches should be fertilized ~30-40 minutes apart.

1. Concentrate eggs (~10 million) in 2 litres FSW, in 10 litre bucket.
2. Fertilize.
3. Mix embryos regularly with plunger.
4. Look for ~50% PB1 formation (~40-60 min).
5. Add CB (contents of 1 vial, 1ml of 1mg/ml in DMSO), mix well, stir regularly
6. Wait 20 minutes, mixing embryos regularly with plunger.
7. Rinse with FSW:
    a. Pour small amounts of embryos (to reduce clogging) onto large diameter 20 µm Nitex screen
    b. Rinse embryos with FSW, then rinse into 10 litre bucket. 2 litres volume total.
8. Add 0.01% DMSO (contents of 1 vial, 0.4ml DMSO), to 10 litre bucket (2 litres volume total).
9. Wait 15 minutes, mixing embryos regularly with plunger.
10. (While waiting, clean the Nitex screens, in preparation for DMSO rinse)

11. Rinse with FSW:
    a. Pour small amounts of embryos onto large diameter 20 µm Nitex screen (to reduce clogging)
    b. Rinse embryos with FSW, then rinse into any size bucket.
12. Pour embryos into 1000 litre tank.

Control Treatments:
1. Concentrate eggs (~10 million) in ~2 litres FSW, in 10 litre bucket.
2. Fertilize.
3. Stir embryos regularly, for ~20-30 minutes.
4. Pour embryos into 1000 litre tank.

Appendix 2: Instructions for making Propidium iodide nucleus staining solution

To make 200ml 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
- 0.2 ml Triton X-100
- 20 ml DMSO
- 179.8 ml ddH_2O

Store refrigerated in a dark container, protected from light.

Appendix 3: Detailed protocols for preparing samples for ploidy analysis via Flow Cytometry

a) Staining Procedure, for Larvae

1. Collect several hundred larvae in a small microcentrifuge tube (e.g., 1.7 ml size tube), 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 lid of tube, and shake vigorously on a vortex mixer for ~20 seconds.
5. Incubate in the dark at room temperature for at least 10 minutes.
6. Shake again on a vortex mixer for ~10 seconds.
7. Screen through 20 µm Nitex mesh (to remove larger bits of tissue and shell), and collect filtrate in a flow cytometer tube.
8. (Alternatively, if the sample does not have many larger bits of tissue, pipette off the supernatant and place directly into flow cytometer tube).
9. Run sample on flow cytometer.

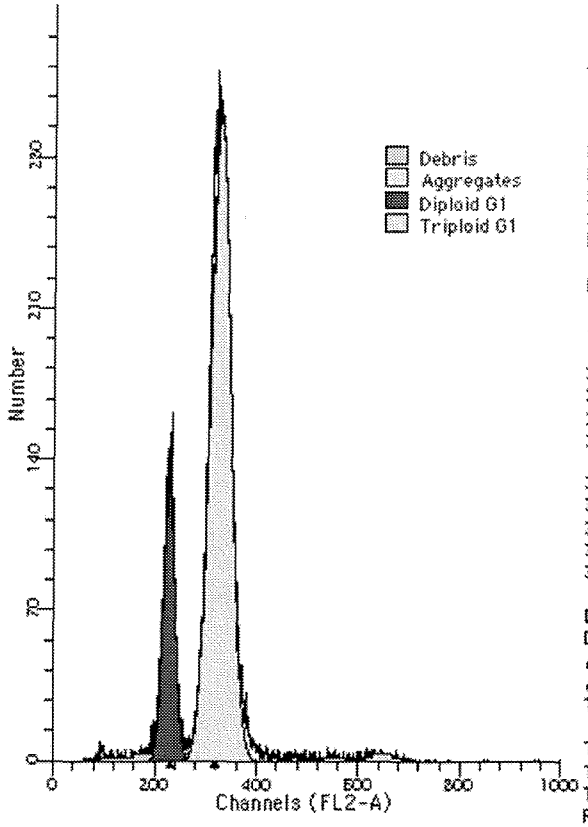
b) Staining Procedure, for Fresh Tissue

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 supernatant seawater, leaving just the pellet of tissue.
4. Add 0.5 ml of Propidium iodide solution.
5. Close lid of 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 sample must be run at a later time, freeze sample now at –70°C).
8. Shake again on a vortex mixer for ~20 seconds.
9. Screen through 20 µm Nitex mesh (to remove larger bits of tissue), and collect filtrate in a flow cytometer tube.
10. Run sample on flow cytometer.

Appendix 4: Sample page output from ModFit ploidy analysis software

Illustrated below is an example of a ploidy analysis as performed on FACSCalibur flow cytometer data by ModFit cell cycle analysis software, available from Verity Software House of Topsham, Maine, and custom programmed for use with sea scallop ploidy data.

LARVAL SEA SCALLOP PLOIDY ANALYSIS
Fall 2002 Trials, Day 22, Wednesday, October 23, 2002



File analyzed: CB-2, Day 22, Run3
Diploids: 19.84 %
Triploids: 80.16 %

Analysis Data

File Location: FACStation G4DP:-Dan's FACSStation Folder:-Dan's FACS Files:SCALLOP PLOIDY TRIALS:Sea Scallops-3n Trial, Fall/02:Day 22, 3n Scallops, Oct 23/02:
File Name: CB-2, Day 22, Run3

Model ID: Test 3n Model
Analysis Type: Manual analysis
Analysis Date: 23-Oct-2002, 15:6:1

2n %: 19.84
2n Peak Area: 3589.9067
2n Peak Mean Channel: 227.86
2n %CV: 4.98

3n %: 80.16
3n Peak Area: 14500.8057
3n Peak Mean Channel: 326.84
3n DNA Index: 1.43
3n %CV: 5.98

Debris %: 4.13
Debris Area: 795.9174
Aggregates % : 2.05
Aggregates Area: 396.1477
Total BAD %: 3.40

Total # of Events: 20000
Total # of Modelled Events: 19283
Total # of Events in Cycles: 18091
Reduced Chi-Square: 1.470

Acquisition Data

Acquisition Date: 23-Oct-02, 14:58:43
Acquisition Software: CellQuest Pro™ 4.0
FACSCalibur Serial# E3850
Total # of Events: 20000

Sample ID: CB-2, Day 22, Run3
Acquisition Panel Name: Scallop Larvae, Fall 2002
Tube Name: tube #3-CB-2
Comments: October 23, 2002 Triploid Analyses
Sea Scallop Larvae, from Oct.1/02 Spawn
22-day old embryos
LO flow rate
20000 events collected in Run 1
20000 events collected in Run 2
20000 events collected in Run 3

Model Fit Diagnostics

4.98	% CV
18091	Cell Number
181	Avg. Cells Per Channel
80.16	Aneuploid Fraction
3.40	% B.A.D.
1.47	RCS

Bedford Institute of Oceanography
Scientific Imaging and Flow Cytometry Laboratory