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TRIPLOID PRODUCTION IN *MYTILUS EDULIS*

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the Department of Health Management

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ABSTRACT

Prince Edward Island (PEI) is a leader in North America in the production of blue mussels *Mytilus edulis*. Concerns in the PEI mussel aquaculture industry over poor product quality during, and immediately after the spawning period have prompted research on triploid mussels. Triploid shellfish are sterile and retain quality during the spawning period. The aim of this research was to develop a method to produce triploids and to test performance of them versus normal diploids under culture conditions.

The first goal of this research was to develop a triploid induction technique and optimize the technique so it could apply to commercial production of seed. This involved investigating the timing of meiotic events in the local blue mussel. Initial results indicated that in order to induce triploidy by blocking the second polar body of the egg, a treatment starting time of 21 minutes post fertilization at 20°C (when 50% of the eggs had extruded polar body 1) was optimal. Poor yields in subsequent spawns caused a reevaluation of this factor by investigating the meiotic events in depth. The resulting description of meiotic events was used to develop a series of suitable treatment time windows for testing in the optimization of the triploid inducing technique.

Triploids were successfully produced with the use of 6-Dimethylaminopurine (6-DMAP) at various concentrations and at various times post fertilization of the eggs. Initial results showed an optimal 6-DMAP concentration range of 300-500 μmol/L. Further optimization, based on the meiotic events previously described, gave an optimal egg treatment of 400 μmol/L of 6-DMAP for 10 minutes following 24 minutes post fertilization at 20°C. This treatment yielded 83.1% triploids in induced samples with a survivorship of 1.39% to the D-veliger stage. Further optimization was not possible within the confines of this graduate program.

Field evaluations of diploid and triploid siblings demonstrated that triploids grew larger than diploids. The growth difference was evident in an area of above average mussel growth after just nine months in the field. A difference between triploids and diploids was not detectable in an area of below average mussel growth until the second year of growth, suggesting a possible variation in the growth of triploids versus diploids related to growout site. Triploids examined after a spawning event showed no histological evidence of spawning, while 71% of diploids showed some evidence of spawning. Relative soft tissue weight, and condition index were higher in triploids. The results demonstrated that triploids retained weight and did not spawn while diploids did. As well, a highly skewed sex ratio confirmed a previous study on *Mytilus galloprovincialis* showing a male dominant sex ratio in triploid mussels.

Triploid mussels appear to fulfill the requirements of the industry in terms of a high growth rate and a lack of spawning compared to sibling diploids. Further optimization of the induction technique and additional production trials are required for industry to fully consider adopting the technology as part of the PEI mussel industry.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

1.1 *Mytilus edulis* – Biology and Life History

The blue mussel, *Mytilus edulis* is one of six species of mussels cultured worldwide. It is the predominant mussel cultured in Prince Edward Island (PEI), Canada. *Mytilus trossulus*, which is interspersed in some small areas, has not been prominent in rope cultured PEI mussels. The true taxonomic difference between the two is the subject of debate (Mallet and Myrand, 1995). The two are virtually indistinguishable visually (Scarratt, 1993) so the difference in percentage of total production is not accounted for in statistics. For the purpose of this thesis *Mytilus edulis* will be considered as the major commercial mussel of importance in PEI.

Mytilus edulis is a filter feeding bivalve mollusc of the family Mytilidae. The external anatomy consists of two valves covered in a dark periostracum. These valves are connected by a rubbery hinge ligament on the upper dorsal side. The internal tissues are suspended by retractor muscles between two valves that can be closed tightly together by adductor muscles when environmental conditions are unfavourable (Scarrat, 1993). Opposite to the hinge on the ventral side the mussel is attached to the substrate by strong threads secreted by the byssus gland. Typical of this family is the blue mussel's muscular foot, which is black in colour and it extends from the shell in order to facilitate movement (along with the byssus and associated muscles). Commonly, small mussels demonstrate significant movement by successively releasing the byssal threads, moving with the foot, and attaching with the byssus once again. The shell is secreted by the mantle, which surrounds the internal soft tissues. The mantle also contains the gonad of the mussel. Mussels are dioecious, that is, they are either male or female, although there are rare occurrences of hermaphrodites reported (Mallet and Myrand, 1995). As the mussel matures the gonad becomes a cream colour to a bright orange. In general males are creamy in

colour and females are, but not always, more orange in comparison (Mallet and Myrand, 1995). Both feeding and respiration are facilitated by water being moved actively from an incurrent siphon, and passed over the ciliated gills. Food, in the form of phytoplankton or organic detritus, is moved along the gills by cilia ventrally and anteriorly, towards the umbo. Near the umbo the food particles are sorted by the labial palps. Rejected particles are discarded from the shell as pseudofeces, while accepted food particles are sorted into the mouth and digestion occurs in the stomach. The stomach contains a crystaline style, a short, rod-like structure that grinds particles and facilitates movement through the digestive tract as it rotates. Food then passes through the digestive tubules where absorption occurs by the digestive cells. Feces are passed out though the anus, near the exhalent siphon.

The factors controlling reproduction of mussels are poorly understood. It is clear that temperature and food supply are important, but it is likely that they interact with other factors such as day length, tidal cycle, and salinity to initiate spawning (Scarratt, 1993). In *Mytilus edulis* a rise in temperature through 10°C to 12°C is generally thought to be essential to spawning (Bayne, 1976; Mallet and Myrand, 1995). High food levels, a physical disturbance such as a storm, or water movement during spring tides are also believed to trigger spawning (Newell *et al.*, 1991; Bayne, 1976). In PEI, mussels generally spawn in late spring or early summer, following the spring ice melt and phytoplankton bloom as water temperatures rise. In some areas spawning can also occur throughout the summer or in the fall, although the spring spawn tends to be more common (Mallet and Myrand, 1995).

Fertilization is external and occurs when the majority of mussels in one area release some or all of their gametes into the water. Fertilization occurs when a sperm successfully penetrates an egg. Egg size when spawned is 68-70 μm (Bayne, 1976), but ranges from 60 to 90 μm

(Gossling, 1992; Scarratt, 1993). Embryonic development begins immediately and within 12 hours the embryo has developed cilia and is a free-swimming trochophore larva. Within 24-48 hours the embryo undergoes a change, secreting a shell and having a characteristic "D" shape. At this point the embryo is called a straight-hinged veliger, D-larva, or D-veliger, measuring approximately 100 to 120 μm (Sprung, 1984; Gossling, 1992). The word veliger is derived from the presence of a velum, which is a ciliated protrusion that enables the veliger to swim and capture food (Scarrat, 1993). The veliger grows for several weeks, losing its characteristic straight hinge as it becomes a later veliger. At approximately 250 μm in size the gut is visible as a dark spot in the animal and a foot becomes apparent and begins probing surfaces (Scarratt, 1993). This stage is called the eyed-larvae, or pedaveliger stage. At this stage the animal is ready to undergo the metamorphosis from a planktonic animal to a sessile benthic one (Bayne, 1976). When a suitable point of attachment is found the pedaveliger secretes byssal threads and undergoes metamorphosis. At this point the newly settled mussel, now referred to as spat, can detach and drift to another more suitable site. This may be done several times (Scarratt, 1993). The mussel then lives as an attached sessile filter feeding organism, and can become sexually mature and spawn as early as the following year.

1.2 Triploidy in Bivalve Molluscs

1.2.1 History

Triploids are organisms with three sets of chromosomes, and are different from most other sexually reproducing organisms that have two sets (diploids). Triploidy is an aberrant genetic state in molluscs, and most organisms, and therefore must be produced artificially. Triploids have larger cell nuclei as they contain more DNA, and consequently have larger cells.

Triploids also do not normally undergo meiosis, as the three sets of chromosomes cannot properly synapse. Therefore triploids have poorly developed gonads and produce far fewer gametes than diploids (Allen, 1988).

Triploidy was first proposed as a genetic manipulation of potential value in aquaculture of fish (Purdom, 1972). The idea at the time was that triploid animals would grow larger simply because they had larger cells, much in the way that polyploid plants do. It was demonstrated however, that this was not the case because the cell number actually decreases when cells are larger, thus triploid fish tended to be the same size as diploid fish (Purdom, 1972). The main benefits derived from triploids come from the fact that they have poorly developed gonads. As gonads are undeveloped, triploids generally provide a more desirable product during the natural spawning season, when reproductive output causes a decline in the quality of diploids, or impedes growth through energy utilization for spawning.

Triploid shellfish were first produced at the University of Maine in the early 1980's. A fungal metabolite being used to produce triploid Atlantic salmon, cytochalasin B, was used to induce triploidy in these first experiments (Allen, 1988). The first triploid shellfish produced was the American oyster, *Crassostrea virginica*, but since that time many different commercially important shellfish have been used to produce triploids using these and other methods. In the 1980's the concept of triploid shellfish was still new, and there were few hatcheries (a requirement for triploid production) on the east coast of North America so the technology was not easily commercialized (Allen, 1989).

At that time hatcheries on the west coast were a well-established component of shellfish culture, as most seed was hatchery-reared. One of the main cultured species on the west coast was the non-native Pacific oyster, *Crassostrea gigas*. This species produces offspring

inconsistently outside of its natural habitat, therefore hatcheries were developed in order to stabilize the supply (Chew, 1984). The Pacific oyster is extremely fecund, and the high degree of reproductive output makes it unmarketable during the spawning season (mid-late summer). As the required large hatcheries were already in place, triploids were addressed as a tool to solve the industry's marketing problem. This work was begun in the early 1980's at the University of Washington, mimicking some of the east coast work. Success, and a large degree of industry interest and support spurred the progression of the technology to the point that it is highly accepted and a high percentage of the Pacifc oysters produced on the west coast today are triploids. In 1994 triploid oysters accounted for somewhere between one third and one half of the total cultured Pacific oysters in the state of Washington alone (Chew, 1994). A more recent estimate of 10-15 billion larvae per year (constituting 25-50% of total oyster production) from the large west coast hatcheries has been given as total annual triploid Pacific oyster production (Chew, 2000) on the west coast. Thus, triploid Pacific oysters can be easily produced in large numbers, and experimental scales can be successfully increased to a commercial scale. It was also proven that the process was economically feasible, and that growers would realize the benefits from producing triploids (Allen, 1989).

1.2.2 Triploid Production Principles

In order to adequately understand triploid production in bivalves it is necessary to fully understand the meiotic divisions in both the sperm (prior to fertilization) and egg (prior to, and post fertilization). A review of meiosis in bivalves follows.

Most higher animals, including commercially important bivalve molluscs, are diploid (Beaumont and Fairbrother, 1991). Diploid cells contain a matching set of maternal and paternal

chromosomes. Mussels, for example, have 2N=28 (14 pairs) chromosomes (Bayne, 1976). Normal cell division, called mitosis, involves the complete replication and division of each chromosome into two identical daughter chromatids. Germ cells however, located in the gonad, undergo meiosis in order to produce gametes. Meiosis consists of two divisions and is the method by which most diploid (2N) animals produce haploid (having one set of chromosomes = 1N) germ cells. During this special type of cell division, germ cells halve their chromosome number so that when combined as gametes (1N) during syngamy (the joining of gametes during fertilization to produce an embryo) they produce an individual with a full set of chromosomes (2N) (Alberts *et al.*, 1989).

The two divisions of meiosis are called meiosis I and meiosis II. In the early stages of meiosis I homologous chromosomes pair together to form a tetrad of four chromatids. It is at this point where genetic recombination takes place due to crossing over of the chromatids. At the end of this division the chromosome number is halved (from 2N to 1N) with one chromosome going to each daughter cell. The second division (meiosis II) follows and is identical to mitosis in that each daughter cell from this division is left with one chromatid from each chromosome, making the daughter cells haploid (1N) (Beaumont and Fairbrother, 1991). The stages of meiosis, in simple terms, are:

a) Meiosis I, consisting of: prophase 1, metaphase 1, anaphase 1, telophase 1, and

b) Meiosis II, consisting of: prophase 2, metaphase 2, anaphase 2, telophase 2.

In shellfish, meiosis results in each male germ cell producing haploid sperm (spermatogenesis). Female germ cells follow the same pattern, however, the first meiotic division is unequal such that most of the cytoplasm is contained in one of the daughter cells (the precursor to the egg, or oocyte). The other smaller daughter cell is called the first polar body.

Meiosis II follows a similar pattern to produce a final oocyte and a second polar body. Neither polar body contributes any chromosomes to the zygote (Beaumont and Fairbrother, 1991). During oogenesis (egg production) however, meiotic divisions do not occur prior to fertilization and the egg is arrested in metaphase I prior to fertilization (Raven, 1966) as most molluscan eggs are spawned at this stage. Meiosis then proceeds after activation by the sperm. Table I.I is a summary of the meiotic events occurring in the egg as they pertain to triploid induction (descriptions adapted from Beaumont and Fairbrother (1991).

Figure 1.1 illustrates spermatogenesis and oogenesis in bivalves, showing the stage at which the resulting male and female gametes are released during spawning.

In spermatogenesis the process begins in the testis with the spermatogonia. From one spermatogonia, successive mitotic divisions produce 64 spermatogonia which develop into first order spermatocytes (Gossling, 1992). These first order spermatocytes, when mature, develop four sets of chromosomes (4N) as the chromosomes double in preparation for the first meiotic cleavage. After the first meiotic cleavage the resulting 128 second order spermatocytes are diploid again (2N). The next meiotic division results in 256 haploid (1N) spermatids, which then go through a maturation to become tailed spermatozoa. Thus, one spermatogonia, after divisions, gives 256 spermatids, and thus 256 spermatazoa (Gossling, 1992). It is at this stage that the male spawns, shedding haploid spermatozoa into the water.

Oogenesis begins in the ovaries with the oogonia. Again, the chromosomes double in preparation for the first meiotic cleavage, producing oocytes (4N). Oocytes are shed into the water at this stage during spawning, and do not develop further until activated through fertilization by sperm. After the first meiotic cleavage the resulting oocytes are again diploid (2N), and have released the first polar body (2N). In some cases this polar body can go through

the second meiotic cleavage, but it never develops beyond that. The oocyte goes through the second meiotic cleavage, releasing the second polar body (1N). The result is a haploid (1N) ootid, which after fusion of the male and female pronuclei, results in a diploid embryo with both a maternal and paternal chromosome set.

Table I.I The stages of egg meiosis and division as they pertain to triploid induction in molluscs.

Meiotic Stage of Egg Development	Important Features
Prophase 1	Prophase 1 occurs prior to fertilization. During this stage the chromosome number in the egg doubles as the DNA replicates. The chromatin condenses and chromosomes become visible.
Metaphase 1	Metaphase 1 is the stage at which development is halted prior to fertilization. Like chromosomes are lined up in preparation for separation during division.
Anaphase 1	Anaphase is initiated after fertilization by sperm. During this stage chromosomes are separated , with one set nearing the egg wall.
Telophase 1	The set of chromosomes nearest to the egg wall buds off with a small amount of cytoplasm during this stage. This bud is known as a polar body, and is the first of two. Polar body 1 removes one half of the female pronucleus. At this time, the diploid female pronucleus and the male pronucleus make the egg essentially a temporary triploid.
Prophase 2	During this stage the chromosome number in the egg does not double. The chromatin again condenses.
Metaphase 2	Similar to Metaphase 1, chromosomes in the female pronucleus prepare for division.
Anaphase 2	Division of the female pronucleus begins.
Telophase 2	A second polar body is produced in the same manner as in telophase 1, producing polar body 2. This division makes the female pronucleus haploid, and is the stage which triploid production seeks to block.
Cell Cleavage	Although not a meiotic stage, just after telophase 2 is complete the female and male pronuclei fuse to form a normal embryo. This embryo then begins growth by the first cell cleavage. Triploid production ideally does not interfere with this stage so as to minimize mortalities.

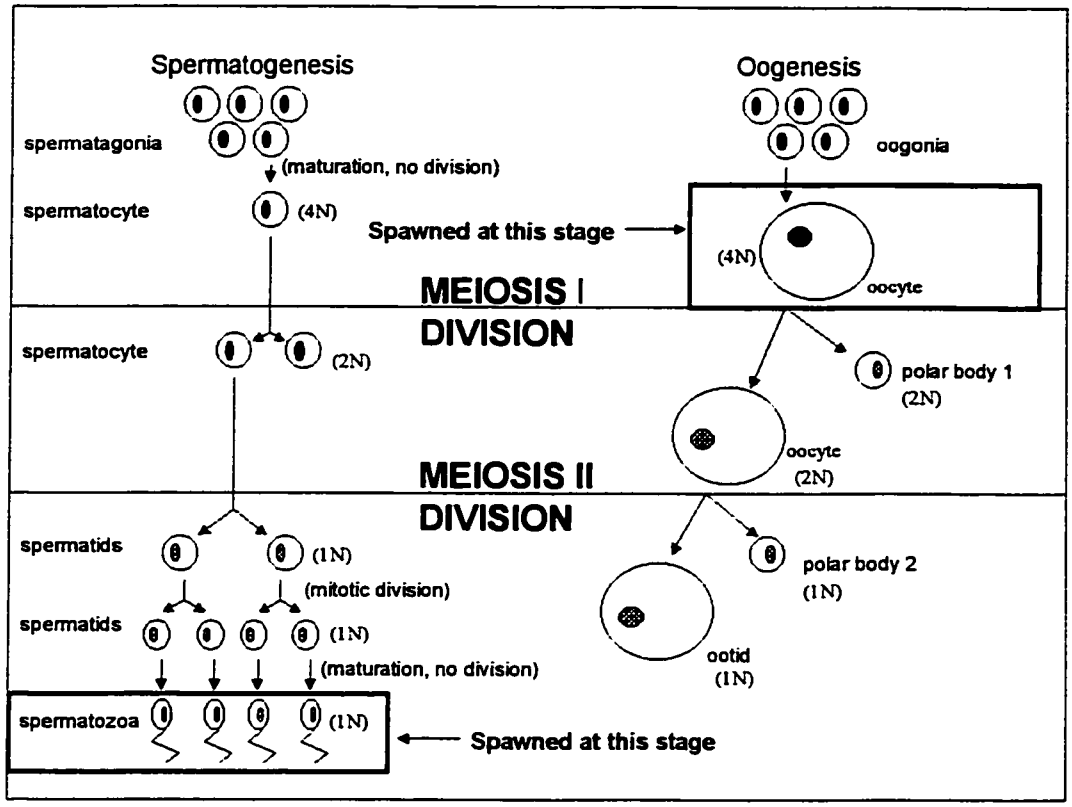

Figure 1.1: General descriptive features of spermatogenesis and oogenesis in bivalves (from descriptions in Gossling, 1992).

1.2.3 Triploid Induction

Figure 1.2 summarizes the meiotic events prior to and immediately following fertilization in bivalves. As illustrated, two polar bodies are shed from the egg after fertilization by two successive meiotic cleavages. Figure 1.3 illustrates the difference in this process when triploids are produced. The blocking of the second meiotic cleavage produces a triploid. If the first meiotic cleavage is blocked, triploids can also be produced as the second polar body will expel the extra 2 sets of maternal chromosomes to produce a triploid. However, the blocking of the first polar body extrusion, in general, results in a lower percentage of triploids and a higher mortality rate than by blocking the second polar body. Therefore, the blocking of the second

polar body extrusion is considered as being more practical (Beaumont and Fairbrother, 1991). Thus, triploid bivalves can best be produced by blocking the extrusion of the second polar body during the second meiotic cleavage of the egg. The result is the retention of the extra set of maternal chromosomes, which combined with single maternal and paternal set, give rise to a triploid. After the polar body extrusion blocking mechanism (most often a chemical) is removed, the egg goes on to divide and develop into an embryo in the same manner as would a diploid.

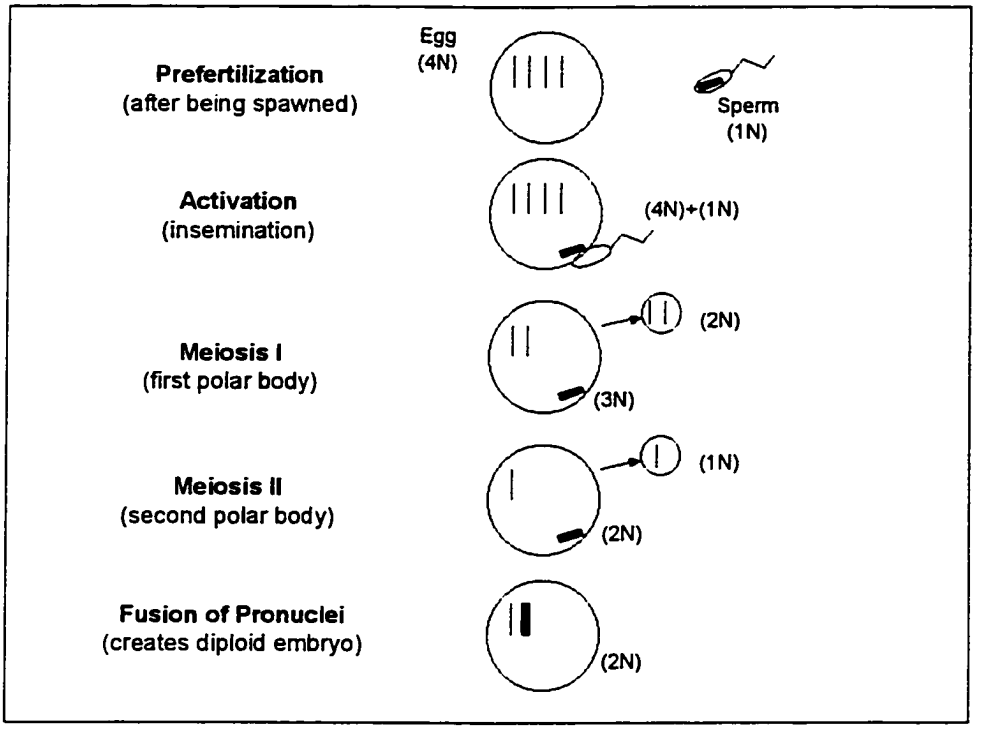

Figure 1.2: Normal meiosis in the mussel egg.

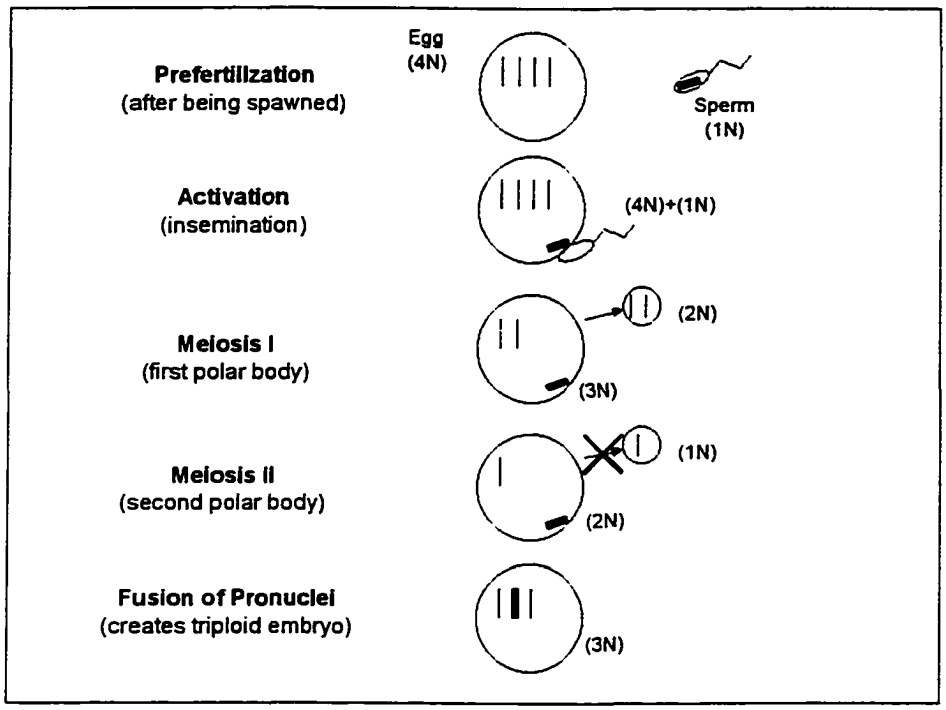

Figure 1.3: Disruption of normal meiosis in the mussel egg to induce triploidy by blocking polar body 2 extrusion.

1.2.4 Blocking Polar Body Extrusion

There have been a variety of investigated methods used for cleavage inhibition to produce triploid shellfish. These have included physiological shocks like heat or pressure extremes and chemical treatments such as calcium, caffeine, cytochalasin B (CB), and 6-dimethylaminopurine (6-DMAP) (Lutz, 1998). All blocking treatments involve applying a treatment for the corresponding amount of time estimated as when the polar body is being extruded. In the case of chemicals or heat treatments, the eggs are simply rinsed and returned to their previous temperature or water treatment, allowing them to resume normal development after treatment.

Pressure treatments have given little success. Heat shocks, but not cold, hold more promise (Allen, 1986; Yamamoto and Sugawara, 1988; Beaumont and Fairbrother, 1991).

Caffeine, and calcium also showed limited success in triploid induction by blocking meiosis (Scarpa *et al.*, 1994). The most promising of all methods to date has been chemical induction by either using CB or 6-DMAP. CB has been the chemical of choice in the past. However, CB is toxic (a carcinogen), and must be dissolved in Dimethylsulfoxide (DMSO) as a carrier due to the fact that it does not dissolve in water. Cytochalasin B has come under the scrutiny of the United States Food and Drug Administration because of its potential danger to hatchery personnel. Health and safety concerns currently restrict its use in oyster hatcheries (Eudeline *et al.*, 2000). The lack of carcinogenicity, cost, and water solubility (Gerard *et al.*, 1994) has made 6-DMAP more heavily investigated in the last decade. Similar yields to CB have been produced with 6-DMAP in most cases (Gerard *et al.*, 1994). Practicality of each chemical may vary with species however, as 6-DMAP gave disappointing results when investigated with the eastern oyster, *Crassostrea virginica* (Scarpa *et al.*, 1995). Both CB and 6-DMAP act through slightly different mechanisms which are discussed in depth in chapter 3, section 3.1. The lack of toxicity, easy handling, as well as the fact that 6-DMAP generally gives good yields make 6-DMAP the choice for triploid production in *Mytilus edulis*.

One further consideration with respect to the blocking of the second polar body (see Figure 1.4 for a microphotograph of a polar body) extrusion is the timing of meiotic events. In order to induce triploidy successfully one must be able to identify the timing of this event so as to accurately block only the second polar body extrusion, allowing subsequent and previous events to go unchanged. This also involves ensuring that most eggs are synchronous in their meiotic events to avoid blocking other stages in a number of the treated eggs. Chapter 2 investigates these factors in preparation for triploid induction trials.

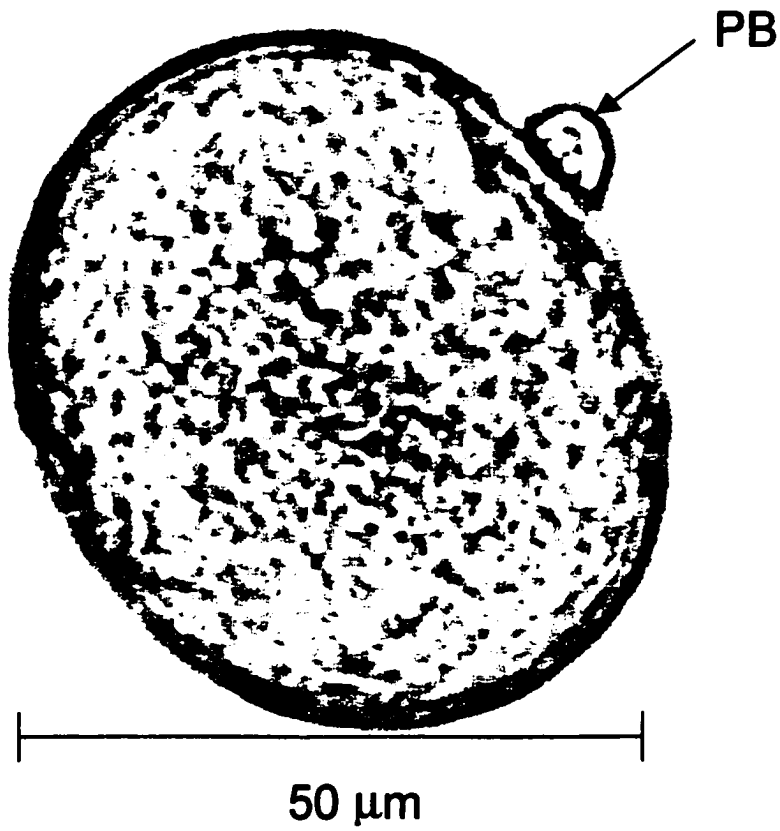

Figure 1.4: Mussel egg showing extruded polar body (PB); light microscopy (100X).

1.2.5 Determining Which Bivalves Are Triploids

There have been a number of ways to verify the ploidy of fish and shellfish since the advent of polyploids. These include chromosome counts, polar body counts, microfluorometry, nuclear sizing by coulter counter or microscopy, electrophoresis, and flow cytometry (Beaumont and Fairbrother, 1991).

Counting chromosomes is the most direct method of verification, and can be used to compare other techniques. It involves fixing eggs in Carnoy's fixative, or taking tissue from an individual and fixing. Colchicine is often used to arrest mitosis in metaphase and make more

metaphase plates available for counting. This is a time consuming method and is not suitable for work when a large sample of individuals must be verified (Beaumont and Fairbrother, 1991).

Counting polar bodies of early embryos has been suggested as a suitable method of verification in some species. This method is limited to verification in early embryos, and although it shows value with the scallop, *Pecten maximus* (Beaumont, 1986), it is unsuitable for the mussel, *Mytilus edulis* due to difficulties in recognizing and recording polar bodies associated with early embryos (Beaumont and Kelly, 1989).

Nucleic acid stained with an aromatic fluorochrome, 4'-6-diamidino-2-phenylindole (DAPI) is excited with ultraviolet light (365nm) and fluorescent intensity is measured using a photometer in the technique know as microfluorometry. The result is a histogram of fluorescent intensity, with peaks distinct peaks associated with haploid, diploid, and triploid cells (Komaru *et al.*, 1988).

Nuclear sizing takes advantage of the fact that triploid cell nuclei have 1.5 times the volume of diploids, and therefore have a greater diameter (Child and Watkins, 1994; Gardener *et al.*, 1996). This technique can be employed with either a coulter counter or microscope, and was first shown to be useful with salmon (Johnson *et al.*, 1984).

Electrophoresis is not used widely to verify ploidy, but was compared with chromosome counting in the soft shell clam, *Mya arenaria*. It was found to have only partial usefulness, as only highly heterozygous loci give useful data (Allen *et al.*, 1982).

Flow cytometry involves staining the nuclei of cells with a RNA-DNA specific dye such as DAPI. In liquid suspension, the stained cells are excited by a laser. They then pass by a cytofluorographic analyser where the intensity of fluorescence is recorded (Allen, 1983). Many tissue types may be used for this procedure. The samples are always run together with a standard

to determine the relative amount to DNA. Flow cytometry has proved to be a fast, accurate procedure, which need not necessitate killing the adult animal (Allen, 1983). Flow cytometry is widely used and currently used by private hatcheries on the west coast of the United States.

Due to the large volume of required samples, and due to the ease, reliability, and accuracy of the results, flow cytometry is the method used in the current study on triploid *Mytilus edulis*.

1.2.6 Current and Past Research Trends With Triploid Bivalves

Triploid research with bivalves can be divided into two main categories: research on how to make triploids, and research on the differences between triploids and diploids. However, many of the past and present studies may fit into both categories.

Once the initial work was done showing that triploid bivalves could be made with the eastern oyster, *Crassostrea virginica* (Stanley *et al.*, 1981), and that they might be beneficial for aquaculture, a number of studies attempted to produce triploids using similar techniques. Beaumont and Fairbrother (1991) give a detailed review of the species researched through the 1980's, citing at least eleven species of bivalves for which triploids have successfully been produced. In the last decade triploid bivalves have become well known and more species have been successfully produced. Virtually any species of commercial value, and even some with little admitted commercial value, have been produced to aid in other studies such as multilocus heterozygosity and size (Beaumont *et al.*, 1995).

As well, studies that have been made on different methods to produce triploids are also prominent in the literature. As mentioned previously, a number of methods have been used to induce triploidy. Scarpa *et al.* (1994) compared 6 different methods to produce triploidy in

bivalves. 6-DMAP has become more prominent for study in producing triploids because of its lack of toxicity, solubility in water, and its high yields (Lutz, 1998). Probably the most significant discovery in this area is that of the production of tetraploids for the subsequent production of 100% triploid offspring without the use of chemicals (Chew, 1994). Guo and Allen (1994) produced the first viable tetraploid oysters for this purpose. Tetraploid oysters went on to be used to produce triploid oysters (Guo *et al.*, 1996). This is done by fertilizing tetraploid eggs with diploid sperm. The tetraploids reproduce normally, and can be crossed with one another to make more tetraploids. The resulting triploids are sometimes called "natural" triploids in the industry. This research is making triploid production more accessible to hatcheries cautious of using triploid inducing chemicals. The fact that 100% triploids are produced each time makes them more useful in the sterilization of exotic species for introduction into a nonnative environment. As well, losses in yields due to toxicity of the triploid inducing technique are eliminated by this method, greatly improving triploid yields (Eudeline *et al.*, 2000). Although useful, tetraploid production is an extremely difficult and time consuming process and was therefore not useful for the current project on mussels.

Triploid versus diploid disease resistance was also investigated with the disease agents *Haplosporidium nelsoni* (MSX) in the eastern oyster, *Crassostrea virginica*, and *Perkinsus marinus* (dermo disease) in the eastern oyster, *Crassostrea virginica*, and Pacific oyster, *Crassostrea gigas*. In both cases the triploids were found to be equally susceptible to the disease when compared to diploids (Matthiessen and Davis, 1992; Meyers *et al.*, 1991). Matthiessen and Davis (1992) did find, however, that triploids, although showing higher prevalences (more triploids were infected) of MSX, initially suffered lower mortalities and had higher condition indices (meaning they were in a higher state of health) than diploids.

Sex determination has also been investigated using triploids because triploids can help elucidate parental genetic control, as triploids have two maternal sets of chromosomes and one paternal. There is a notable alteration of the sex ratio in some triploid bivalves. All triploid soft shell clams were found to be female (Allen *et al.*, 1986); in contrast, all triploid Mediterranean mussels were found to be males (Kiyomoto et al., 1996). A recent study has also shown the suppression of functional hermaphroditism in the catarina scallop, *Argopecten ventricosus*, causing a gradual replacement of the male gonads. The result was that 96% of all triploids could only be described as female, whereas all diploids remained hermaphrodites (Ruiz-Verdugo, 2000). By contrast, dwarf surfclam and Pacific oyster show no significant difference between sex ratio in diploids and triploids (Guo and Allen, 1994; Allen and Downing, 1990). These findings have led to the proposal of several different genetic mechanisms for sex determination amongst the different investigated species.

The issue of the relative sterility of triploids when compared to diploids, and its relevance to aquaculture has been extensively studied. Initial work showed that triploid oysters could ensure a year-round supply of product (Allen, 1988), and that consumers preferred them over ripe diploids during the spawning season (Allen and Downing, 1991). Severely retarded gametogenesis and increased glycogen levels during the spawning season, compared to diploids, have been reported (Allen and Downing, 1986; Allen *et. al*, 1986; Lee, 1988; Allen and Downing, 1990). Production of sterile oysters that maintain a positive energy balance during the reproductive season and can be marketed all year round is the main commercial aim of triploidy induction (Allen, 1988). Relative fecundity varies in different triploid bivalve species, however, all have very low gamete production and are, for all practical reproductive purposes, sterile (Allen, 1987).

Lastly, many triploid studies involve the evaluation of growth rates of triploids, as in almost all cases triploids have been shown to have superior growth rates when compared to diploids (Guo, 1999, Guo *et al.*, 2001). Table I.II is taken from Guo *et al.* (2001), and summarizes the studies to date on growth differences due to triploidy. To date there have been at least 12 species of bivalves for which growth differences between diploid and triploid adults have been shown. Triploids grow faster than normal diploids by 30-50% in most species, and up to 70-100% in other species. Only in one species were triploid shellfish smaller than diploids (Mason *et al.*, 1988), a result that has not been repeated (Guo *et al.*, 2001).

Table I.II: Percent increase in body size of triploid shellfish over their diploid controls (Guo *et al.*, 2001).

Species	Increase (%)	Age (yr)	Source
Eastern Oyster, *Crassostrea virginica*	41	3	Stanley *et al.*, 1984
	30	2	Barber and Mann, 1991
European Flat Oyster, *Ostrea edulis*	60	1	Hawkins *et al.*, 1994
Dalianwan Oyster, *Crassostrea talienwhanensis*	52	1	Liang *et al.*, 1994
Pacific Oyster, *Crassostrea gigas*	30	1	Guo *et al.*, 1996
Sydney Oyster, *Saccostrea commercialis*	41	2.5	Nell *et al.*, 1994
Pearl Oyster, *Pinctada martensii*	44	2	Jiang *et al.*, 1991
Bay Scallop, *Argopecten irradians*	36	1	Tabarini, 1984
Catarina Scallop, *Argopecten ventricosus*	104	1	Ruiz-Verdugo *et al.*, 1998
Nobilis Scallop, *Chlamys nobilis*	40	1	Lin *et al.*, 1995
Dwarf Surfclam, *Mulinia lateralis*	72	0.3	Guo and Allen, 1994
Hard Clam, *Mercenaria mercenaria*	08	4	Eversole *et al.*, 1996
Softshell Clam, *Mya arenaria*	-15	1	Mason *et al.*, 1988
Average	**42**		

1.3 Mussel Aquaculture in PEI

PEI is a leader in rope cultured mussels in Atlantic Canada and North America. Mussel aquaculture in PEI is similar to that of the rest of Atlantic Canada. All mussel culture in Atlantic Canada is facilitated using suspended culture using a longline system (Mallet and Myrand,

1995). Mussel aquaculture in Atlantic Canada begins with the collection of wild seed mussels in the early summer after a spawning event. In a given area mussels are monitored for a sudden loss of weight, or water is sampled for the presence of mussel larvae. Once a sudden drop in weight is noted or if larvae are present in the water, there is generally a one-week delay before seed collection begins (Scarratt, 1993). Mussel larvae set at approximately 250 μm (Mallet and Myrand, 1995), so proportions of these sized larvae and growth of the local larval population can give accurate accounts of when the major set will occur. There are a wide variety of substrates used to facilitate mussel settlement (Mallet and Myrand, 1995), however in PEI, polypropylene rope, old and frayed from use in the lobster fishery, is predominantly used as a collector. This allows a suitably large surface area for wild seed to set (attach using byssal threads). These ropes are suspended off the backline of the longline system and weighted down using metal spikes or small lead weights. The rope collectors are deployed approximately one week prior to the prediction of spatfall. This is done to allow the ropes to "season". Seasoning is done because it has been found by growers that, in general, more mussel spat attach when the rope collectors have been able to soak for several days in seawater. Spat lines are monitored during the summer and additional buoys are applied as the spat grows and becomes heavier. As well, lines that become infested with starfish may be treated in a solution of hydrated lime and seawater to prevent this predator from consuming the seed mussels.

In the fall the seed collectors are harvested and stripped off. The seed mussels are then anywhere from 5-30 mm in length (Mallet and Myrand, 1995). The seed mussels are then graded by size using a mechanized system, and suitably large mussels are placed in plastic mesh netting tubes called "sleeves", or "socks", also by various mechanized and unmechanized methods. Sock length varies and is dependent on the water depth of the growout site. Seed densities vary within

the mussel socks but are roughly 500-600 mussels per meter of sock (Mallet and Myrand, 1995). Mussels then grow and are harvested when they reach market size. Both the growth time and desired market size varies with site.

In PEI, growers take advantage of the natural life cycle to grow mussels in an aquaculture setting. Figure 1.5 shows the major life cycle stages as they pertain to culture of the blue mussel, *Mytilus edulis*, in PEI. The mussels spawn in the spring or early summer, producing ciliated trochophore larvae within 24 hours. Shortly after the larvae begin to secrete a shell and become D-veliger larvae. The mussels then grow to become larger veliger larvae until they are ready to set and metamorphose. At this stage they are referred to as pediveliger larvae, due to the presence of a foot. The pediveligers set on the spat collector lines within 21-28 days and grow for 4-6 months. These collector lines are then harvested, the mussels are then socked, and deployed again. These grow to market size and are harvested generally in 12-18 months, or more, depending on the specific site growth characteristics.

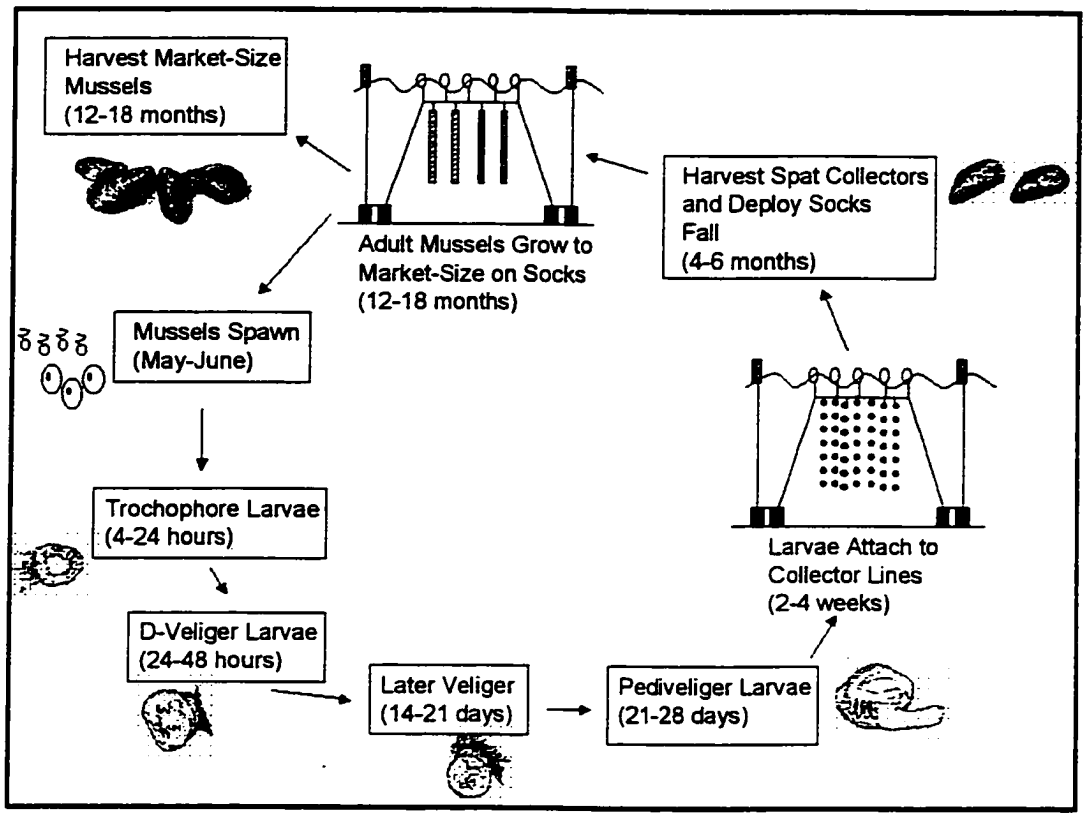

Figure 1.5 Commercial production cycle of *Mytilus edulis* in PEI (adapted from Mallet and Myrand, 1995).

The PEI mussel industry has developed into a successful aquaculture industry in recent years. In 2000 PEI produced 84 % of Canada's total production by weight and 79% by value. Figures 1.6 and 1.7 show growth in the industry since 1986, illustrating the landed value and weight respectively of cultured blue mussel in PEI and the whole of Canada. In the past decade, from 1990 to 2000, PEI has seen a 664% increase in production by weight and an 811% increase by value. There are currently over 1500 jobs directly supported by the industry, many of them full time. Over 10,700 acres are currently leased on 279 separate mussel leases. The majority of the product is marketed as a live fresh product, with markets extending to all of North America and periodically into Europe.

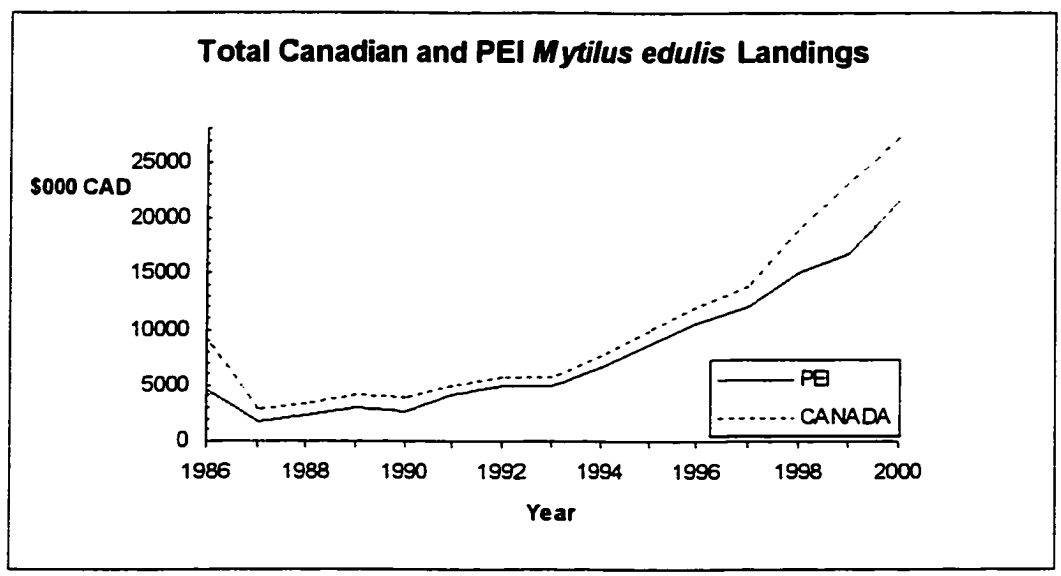

Figure 1.6: Total Canadian and PEI *Mytilus edulis* landings by value (Source: DFO statistics, 2001).

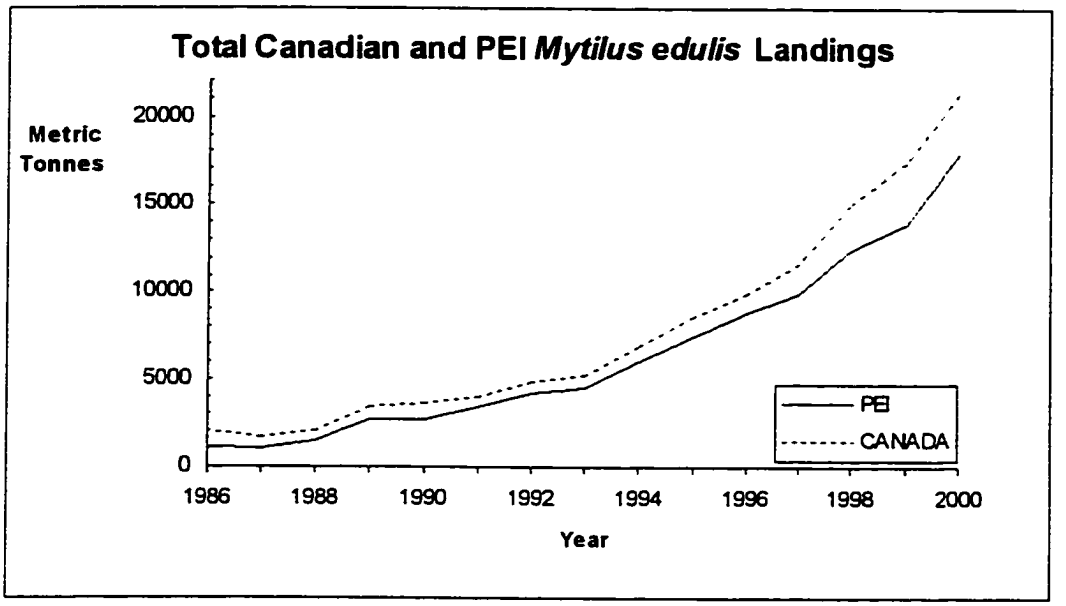

Figure 1.7: Total Canadian and PEI *Mytilus edulis* landings in metric tonnes (Source: DFO statistics, 2001).

1.4 Triploids as a Solution to a PEI Mussel Industry Problem

The PEI mussel aquaculture industry has demonstrated remarkable success in the past twenty years and has become a very significant contributor to the local economy. With effective methods of production established, and the majority of available water leases secured, the mussel industry is now looking for the means to remain competitive in the market place. One area of concern to the industry is the harvesting and marketing of mussels during and just after the spawning season. Ripe mussels (near spawning) can spawn due to the stress of shipping. Mussels that spawn while in transit to market can be returned to the processors for refund. This can also result in dissatisfied customers because of an undesirable odour, a decreased shelf life, and poor quality. Mussels that have recently spawned have significantly reduced meat yield making them less appealing to the consuming public. The marketing of triploid mussels during

this period would alleviate these problems and allow the industry to market a quality product year round. In December 1996 the PEI Cultured Mussel Growers Association identified *"Evaluating techniques to produce triploid mussels to supply markets during the natural spawning season"* as one of the industry's research priorities.

Triploids have poorly developed gonads, produce far fewer gametes (sperm and eggs), and are much less likely to spawn than regular mussels. They reallocate their energy towards growth instead of gamete production. This allows for a more desirable product in triploids while the normal diploid mussels are ripe or spawning.

The development of triploid mussels can be considered as adding value to the existing mussel industry. The issue of spawning during shipment of diploids and low meat yields immediately following spawning continues to be issues with the industry despite various management techniques and procedures to avoid these occurrences. Some PEI processors are investigating opportunities in other provinces to supply the markets at times when PEI mussels are in spawning condition.

The commercial application of the development of triploid mussels could be great. The industry would be able to provide the market with triploid mussels during the natural spawning season thus ensuring a quality product year round to the market. Triploid harvesting and marketing could occur in late May to late June, requiring an estimated 1350-1800 metric tonnes yearly based on the 2000 harvest figures.

1.5 Study Objectives

There are two main objectives to this study. The first is to develop and optimize a triploid inducing technique for the blue mussel, *Mytilus edulis*. The second objective is to evaluate the

performance, in a number of environments (culture conditions), of triploid and diploid siblings with respect to spawning and growth differences.

The thesis is divided into 5 chapters. Chapter 1 is the introduction of the concept and objectives. Chapter 2 introduces the initial work used to develop a triploid induction technique, and to allow for its optimization by looking at the timing of meiotic events as they pertain to triploid induction. Chapter 3 contains the work on the induction of triploidy, and the optimization of the technique. Chapter 4 contains the results of field trials to evaluate gametogenesis as an indication of spawning ability in triploids and diploids. It also addresses the growth differences between diploids and triploids in the field. A short concluding chapter (Chapter 5) follows and summarizes the body of work, assessing the potential for utilization, and identifies areas that should be addressed in future work.

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2. THE DETERMINATION OF THE TRIPLOID INDUCTION INITIATION TIMING

2.1 Introduction

The goal of commercial triploid induction of mussels is to produce a high percentage triploid cohort with a high survivorship throughout the hatchery stage (Allen *et al.*, 1986). Imperative to the induction of both a high percentage triploid and a high survival rate cohort of triploid mussels is the determination of the proper time at which to induce triploidy. In the past, the stage of development when 50% of the eggs showed polar body formation has been used as a common cue to start triploid induction treatment in oysters (Allen *et al.*, 1989). The objective of this study was to determine the optimal time for triploidy induction initiation in *Mytilus edulis* embryos using two methods; the first was based on polar body extrusion and the second on meiotic events. The objective of the first experiment, based on polar body extrusion, was to determine the time post fertilization, when 50% of the embryos developed polar bodies. The second experiment, based on meiotic events, was undertaken to determine the time post fertilization when 50% of the embryos were in Anaphase 2 and the other 50% were in Telophase 2.

In molluscs, triploid induction occurs through the use of an external treatment on a fertilized egg. This treatment has a finite duration, the optimal time of which is determined by the timing of meiotic events. This time from which a triploid induction treatment may be initiated until it is no longer effective may be referred to as the induction window. This chapter will describe the two experiments designed to evaluate the timing of the start of this induction window. This work is the foundation for optimization of the triploid inducing technique in *Mytilus edulis*. Further work on the length of this window (when it is most suitable to end the treatment) and treatment level will be discussed in chapter 3.

Dèsrosiers *et al.* (1993) suggested that triploid induction treatment using 6-DMAP was best at the Metaphase 1 stage. Vadapolas (1999), however, found that more success was gained by treating when approximately 50% of the eggs were in Anaphase 2 and the other 50% were in Telophase 2. Therefore, the purpose of the second experiment was to find the optimal stage, by timing, for future induction refinement.

2.2 Materials and Methods

Both experiments were conducted at the Ellerslie Shellfish Hatchery (ESH), in Ellerslie, PEI was the facility where the experiment was conducted. Microscope slides were subsequently read at the Atlantic Veterinary College (AVC), University of Prince Edward Island (UPEI), in Charlottetown, PEI.

2.2.1 Experiment #1 - The Determination of Polar Body Extrusion Timing in *Mytilus edulis* Broodstock

Broodstock were obtained in November of 1998 from a mussel lease located in Lennox channel, near Lennox Island, PEI. These broodstock were found to be in poor spawning condition, a conclusion reached after observation of several sacrificed individuals which were histologically examined using a light microscope. Approximately 200 of these animals were conditioned for six weeks using a chiller system which utilized recirculated water in an insulated container. During this time they were slowly acclimated from the ambient temperature of 2°C to 8°C. During conditioning the animals were batch fed a mix of cultured microalgae (diatoms and flagellates) during water changes every 2 days.

Spawning

Protocols were developed so as to reduce the possibility of sperm contacting the eggs causing fertilization prior to the experiment commencement and during each stage of the spawning and experimental practices outlined in this chapter. Each container or object that came into contact with the gametes was rinsed with a 5% bleach solution and then fresh water before spawning and between each step throughout the duration of the experiment. On the day of the experiment the animals were taken from the conditioning area, rinsed in fresh water, placed in a 20 L empty bucket and shaken for approximately 2 minutes in order to induce an initial mechanical stress as a spawning cue prior to the actual spawning. They were then placed in individual 1L plastic beakers with approximately 600 ml of 1 μm filtered sea water (FSW) at 18°C. Spawning was induced by a thermal shock of an increase of 10°C, as the animals were taken from 8°C water prior to spawning. This method, is one of those reported to be extremely successful in commercial and research scale spawning practices with *Mytilus galloprovincialis* in the Pacific Northwest (Davis, pers. comm.). After individuals were spawned, egg quality was evaluated microscopically and suitable (mature) eggs were pooled from 3 individuals. Approximately 3 million eggs were used for each temperature treatment. Suitable sperm was also pooled from 4 individuals. Caution was exercised in order to prevent contact between the eggs and sperm prior to controlled fertilization, keeping both separate to reduce this risk of contamination. Fertilization occurred within 2 hours of gamete collection so as not to compromise the quality of the gametes, which diminishes with extended time after contact with seawater. A target sperm-egg ratio of approximately 5 to 25 sperm per egg is desirable in hatchery mollusc fertilization (Davis, pers. comm.). In both temperature samples (15°C and 20°C) the observed sperm-egg ratios fell within this range.

Experiment

It was decided that both temperatures of 15°C and 20°C would be investigated as they are both common, and easily achievable working temperatures in shellfish hatcheries. 20°C was selected as it is a commonly used temperature in most shellfish hatcheries, and 15°C was selected as it is closer to the temperature during the natural local spawning in this species and also common in many shellfish hatcheries. Pooled eggs from the individually spawned females were placed on a 20 μm sieve that was placed in a 20L bucket of FSW. This bucket was placed in a 20°C water bath to keep the eggs and surrounding sea water at that temperature. Sampling of the eggs was facilitated by simply raising the screen out of the water and aspirating eggs from the screen, without an excess of sea water, and immediately returning the screen to the bucket. The eggs were immediately placed in a pre-loaded 2 ml centrifuge tube containing 2% buffered formalin fixative. The sample was mixed well so as to fix the eggs rapidly. The experiment involved fertilizing the eggs, mixing well during this process, and sampling them prior to fertilization, at fertilization, and at 3minute intervals up to the 2-hour mark. Simultaneous to this sampling, the same procedure was being followed using the same egg and sperm pool with a water bath of 15°C.

Scoring Polar Body Extrusion

Polar bodies are visible using light microscopy. In order to aid in detection, the use of a 0.5% aceto-orcein stain was employed. This stain consisted of 0.5% orcein in a solution of 60% acetic acid. This procedure is used specifically for molluscs, as described by Guo (1991). This methodology was based on the original procedure first described by Darlington and La Cours (1962). Samples of eggs were removed from the fixative and placed on a clean microscope slide.

Two or three drops of stain were then added to the slide and a coverslip was added. After allowing the stain to set for two minutes the eggs were viewed and the number of eggs out of the first 50 seen on the slide demonstrating polar bodies were recorded. When this number reached 25 (50% of eggs), the target had been reached and no further samples were required.

2.2.2 Experiment #2 – The Determination of Meiotic Events in *Mytilus edulis*

Broodstock

Broodstock were again obtained in December of 1999 from a mussel lease located in the Lennox Island, PEI vicinity. These animals were also conditioned using a chiller system and recirculated water in an insulated container for 6 weeks. During this time they were slowly acclimated from the ambient temperature of 0°C to 8°C. During conditioning, the animals were batch fed a mix of diatoms and flagellates during water changes every 2 days.

Spawning

Spawning was performed in the same way that it was in Experiment #1. In this experiment however, 4 females were pooled for use, giving approximately 3 million eggs, and sperm from 7 males was pooled for use. The number of males and females used was determined by the number of viable male and females successfully spawned. In this case, 4 females producing high quality eggs and 7 males producing high quality sperm were successfully spawned. Sufficient sperm order to attain a reasonable sperm amount (5-25 per egg) was used. This was determined by a visual approximation of the number per ml and mathematically determining the amount of sperm to add.

Experiment

This experiment was only performed on one sample of pooled eggs held at 20°C, with no other temperature replicate. Otherwise the experiment was the same as that of Experiment #1 except that samples of eggs were taken at every minute from fertilization until the one hour mark. This was done in order to give a more accurate picture of the meiotic events up to egg cleavage, which is reported to occur within the first hour after fertilization (Rattenbury and Berg, 1954; Strathman, 1987; Lutz *et al.*, 1991).

Categorizing of Meiotic Events

Samples were stained using the same procedure and aceto-orcein stain used in Experiment #1. The stained sample was then viewed under a light microscope. Each of the first 50 eggs observed for every sample were categorized as being in a particular stage of meiosis. In the cases where no clear stage was visible, that egg was replaced by the next visualized such that the first 50 readily scored comprised the sample for each time sampled.

2.3 Results

2.3.1 Experiment #1 - The Determination of Polar Body Extrusion Timing in *Mytilus edulis*

Table II.I is a summary of the results. In the 20°C water bath group, polar bodies appeared as early as 15 minutes post fertilization and showed the desired score of 50% polar body display at 21 minutes post fertilization for 2 replicates and 24 minutes post fertilization for one replicate. Samples taken from the 15°C water bath showed a slower development of polar bodies, with none appearing until 18 minutes post-fertilization. All samples from the 15°C water bath showed 50% polar body display by 27 minutes post-fertilization, with one of the replicates

showing this score at 24 minutes. The 15°C water bath samples also showed less synchrony amongst themselves, with replicates showing a discrepancy of as much as 16% (of eggs showing polar bodies) between them at 21 minutes post-fertilization. The largest discrepancy between any replicates in the 20°C water bath sample at any given interval is 6%.

Table II.I: Polar body extrusion in *Mytilus edulis* as determined by light microscopy.

	Percent of observed eggs showing polar bodies							
	15°C				20°C			
Time (minutes post fertilization)	Replicate 1	Replicate 2	Replicate 3	95% Confidence Interval	Replicate 1	Replicate 2	Replicate 3	95% Confidence Interval
0	0	0	0	-	0	0	0	
3	0	0	0	-	0	0	0	
6	0	0	0	-	0	0	0	
9	0	0	0	-	0	0	0	
12	0	0	0	-	0	0	0	
15	0	0	0	-	4	2	2	2.67±1.15
18	2	4	0	2±2	24	26	20	23.33±3.05
21	42	22	26	30±10.58	52	54	46	50.67±4.16
24	40	24	52	38.67±14.05	-	-	68	-
27	74	52	-	63±15.56		-	-	-

2.3.2 Experiment #2 – The Determination of Meiotic Events in *Mytilus edulis*

Figure 2.1 is a summary of the results. Telophase 1, the stage when polar body 1 is extruded, was visible as early as 12 minutes post fertilization. Anaphase 2, the stage that is a precursor to polar body 2 being extruded, began at 22 minutes post fertilization. Telophase 2, during which polar body 2 is extruded, began at a time of 27 minutes post fertilization. Egg cleavage, the first mitotic embryonic cleavage, began at 44 minutes post fertilization. The target time, when approximately 50% of the scored eggs were going through Anaphase 2, and approximately 50% of the scored eggs were going through Telophase 2 was found to be 32 minutes post fertilization.

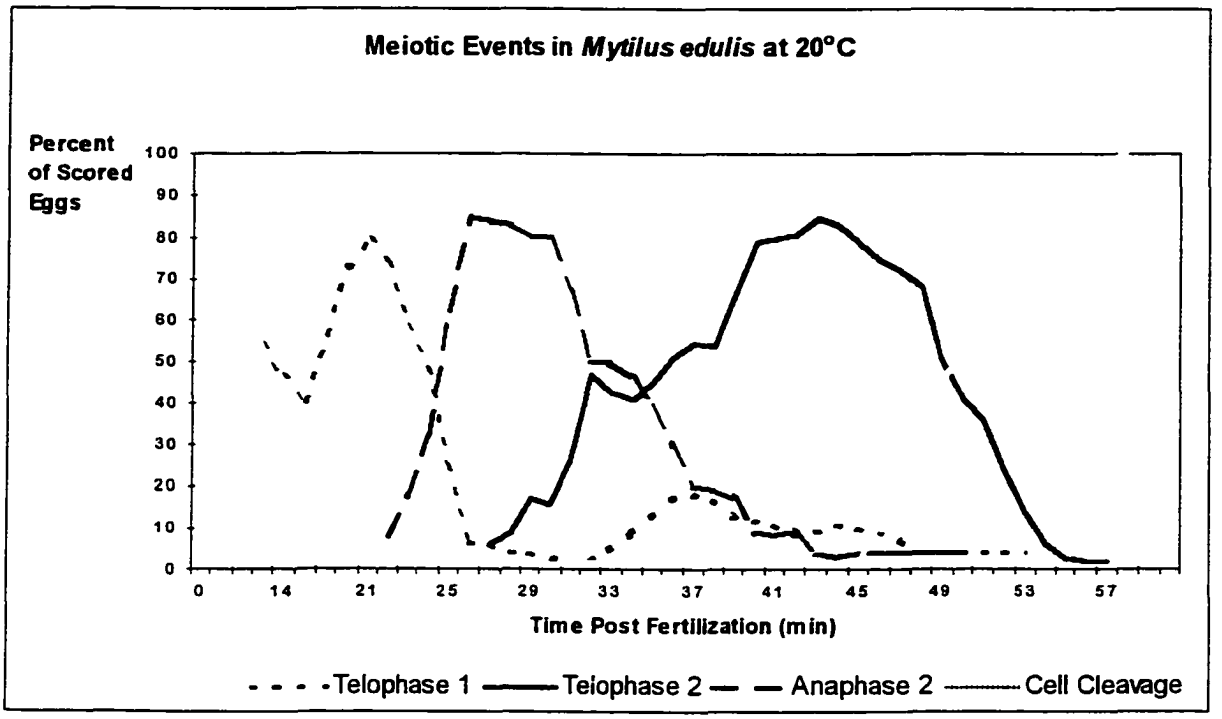

Figure 2.1: Summarized meiotic events in *Mytilus edulis* at 20°C.

2.4 Discussion

2.4.1 The Importance of The Induction Initialization Timing

The determination of the proper induction window (time of treatment initiation to treatment terminating) is crucial to triploid induction for a number of reasons. First, the induction treatment is potentially lethal to fertilized eggs when exposure times are too long. Dèsrosiers *et al.* (1993) found that longer exposures to 6-DMAP interfered with first cleavage and resulted in developmental abnormalities, especially in *Mytilus edulis*. In cases where the induction comes early, although a cohort with a high percentage of triploids might be produced, the survivorship would be low, making the cohort less useful from a commercial perspective. Secondly, the timing might not only lower survivorship, but also affect the percentage of triploids that would be produced in a given cohort. If the induction window, although the proper length of time when

started and ended at the appropriate times, is too late, meiosis II will not be blocked and the production of mostly diploids will result. This would result in a cohort with a high percentage of diploids, and thus a lower percentage of triploids. It is important to note that not all eggs are in the same meiotic stage at any time. Thus, it is important that most of the eggs are highly synchronous in terms of meiotic events, and that they are treated such that most eggs will receive the proper amount of treatment in order to induce triploidy. Lu (1986) found that meiotic events of the eggs are more synchronous at higher temperatures (not exceeding the physiological limits); that is, the meoitic events transpiring in the eggs are in greater unison. Given this finding, a higher temperature within the physiological limit would be expected to produce more synchronous meiotic events. This was supported by the finding in experiment #1 that more eggs were in the same meiotic stage on average at a given sampling time in the 20°C sample compared to the 15°C sample. Salinity also affects timing, but less than temperature does, and not within the range that shellfish would normally be subjected to in a hatchery (Allen *et. al.*, 1989). If the induction window begins too early, the result would be a blocking of meiosis 1 for most of the eggs, resulting in pentaploids (5N) being produced. Pentaploids do not survive to the D-veliger stage (see chapter 1, section 1.1 for life cycle and description of this larval stage). This was shown to occur in *Mytilus galloprovincialis* by Scarpa *et al.* (1993). The authors exposed eggs to 1mg/L of cytochalasin B from 7 to 35 minutes after fertilization, blocking meiosis I. The resulting developing pentaploid eggs showed a subsequent inhibition of first cleavage and died. Therefore the resulting cohort would have a large percentage of triploids at the D-veliger stage, as whatever wasn't a pentaploid would likely be a triploid (having meiosis I completed and being in meiosis II). This cohort would likely have a lower survivorship however, as the pentaploids would all die.

2.4.2 Rationale of The Two Experiments

Experiment 1 was the first attempt to find a starting time for triploidy induction with reference to the time of fertilization. The target of concern was the time at which 50% of all eggs had undergone polar body 1 extrusion. It was assumed at that time that it was more critical to find the initiation time of induction, as the exact duration of the treatment could be refined in further experiments. It was also decided that both temperatures of 15°C and 20°C would be investigated as they are both common, and easily achievable working temperatures in shellfish hatcheries (Davis, pers. comm.), and they would give some insight as to a temperature effect in the induction window. The main objective of this experiment was to determine the time at which 50% of fertilized eggs developed polar bodies. Fifty percent polar body formation has been used as a common cue to start triploid induction treatment in oysters. Therefore, the purpose of this experiment was to find this onset of induction time post fertilization of eggs. This (50% of eggs showing polar bodies) has traditionally been the time when triploid induction treatments had begun for shellfish, ending after various time periods depending on the species (Baker, 1996; Allen *et. al*, 1989).

One challenge to the production of both triploid and diploid animals for growth trials was an unexpected high mortality level during the repeated spawns in the summer of 1999 (see chapter 4, section 4.2.1). This initiated a reevaluation of the treatment window, particularly the induction initiation timing as determined to be between 21 and 24 minutes post fertilization in experiment #1. One possible reason for repeated low survivorships, less than 0.01% to the D-veliger stage, may have been that initiation of treatment to induce triploidy might have been too early. If this was the case, polar body 1 extrusion would be blocked in most eggs, forming pentaploids. Pentaploids do not survive to the D-veliger stage. In turn, however, any eggs that

were past this stage would likely have polar body 2 extrusion blocked and thus become a triploid. The trend in spawns was in concordance with this as the survivors of these spawns, though few, were mostly triploids (all over 80% triploid, with many over 90%).

Therefore, experiment #2 involved a more in-depth study of the meiotic events in *Mytilus edulis* at 20°C, the temperature that showed the most meiotic synchrony in experiment #1. As opposed to attempting to treat at the time just after polar body 1 was extruded, a more finite point was used. This strategy was shown to be successful in Geoduck clam triploid induction (Vadapolas, 1999). Dèsrosiers *et al.* (1993) found that triploid induction treatment using 6-DMAP was best at the metaphase 1 stage. In Geoduck clam induction however, Vadapolas (1999) found that extrusion blocking with 6-DMAP just at the point where 50% of the eggs were in Anaphase 2 and 50% of the eggs were in Telophase 2 produced the best results. Therefore, experiment #2 was performed with this as the target outcome.

Experiments #1 and #2 were in agreement with one another in terms of polar body 1 extrusion. Experiment #1 illustrated that most eggs had extruded, or were in the process of extruding polar body 1 by 21-24 minutes post fertilization. Experiment 2 similarly showed that most eggs were undergoing polar body 1 extrusion, scored as Telophase 1, at a time of 24 minutes post fertilization. The fact that both experiments showed a similar time of polar body 1 extrusion infers that if the induction initialization was in fact early, causing mortalities, it was not because the target of first polar body extrusion was improperly identified. It is possible that a more accurate treatment induction initiation was required, as the one researched and obtained in experiment #2.

Therefore, further optimization can be facilitated and explained by this meiotic model developed in experiment #2. As previously mentioned, the target treatment initialization

researched in experiment #2 was the time when 50% of eggs were going through Anaphase 2 and the other 50% were going through Telophase 2. Further optimization of the induction treatment window (see chapter 3), including the initialization, was based upon this target of 32 minutes at 20°C.

2.5 References

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3. THE DEVELOPMENT AND OPTIMIZATION OF TRIPLOID INDUCTION IN *MYTILUS EDULIS*

3.1 Introduction

To date there have been numerous methods used in attempts to develop triploid bivalve molluscs. Combinations of chemical induction and heat shocks have been the most effective (Chapter 1, section 1.2.4). Once the determination of the triploid induction timing was determined (Chapter 2, section 2.4) the next phase in the production of a high percentage, high survivorship triploid mussel cohort was the development of a strategy for the induction of triploidy (i.e. which method(s) to test) and the optimization of that method. The objective of this study was to develop and optimize an induction strategy.

The results of chapter 2 showed that at 20°C there was a higher degree of meiotic synchrony than at 15°C. This was evident in the results of experiment #1 which showed that a higher percentage of the eggs were in the same meiotic stage at a given time when compared with the 15°C treatment. These results also indicated that at 21 minutes post fertilization, the meiotic score generally associated with optimal triploid production occurred. In experiment #2 of chapter 2 a more comprehensive description of meiotic events in the mussel egg was reported. These results showed that the results of the previous experiment might have suggested an induction starting time that was too early, and that a time of 24 minutes post fertilization may be more optimal.

Induction of triploidy is best facilitated by the chemical-heat shock combination, by the mechanism of blocking of polar body extrusion in the second meiotic cleavage in bivalve molluscs (Beaumont and Fairbrother, 1991; Lutz, 1998). Both chemical concentration and chemical type are important in triploid induction success. Two chemicals have been found to be the most successful in the induction of triploidy in bivalve molluscs. The first, cytochalasin B,

has been the most widely used (Beaumont and Fairbrother, 1991; Chew, 1994; Lutz, 1998; Chew, 2000, Eudeline *et al.*, 2000). Cytochalasin B (CB) is a chemical in a class of fungal metabolites, and inhibits microfilament formation in cells (Copeland, 1974). According to Longo (1982), CB prevents the development of a cleavage furrow in the cell at the base of the protrusion of the polar body. This results in the retention of the set of chromosomes that would have formed the polar body. Cells exposed to CB show neither the gross morphological presence of a cytoplasmic cleavage furrow, nor the filaments of the contractile ring required for effective cytokinesis (Copeland, 1974). CB is hydrophobic and is therefore dissolved in dimethylsulfoxide (DMSO) as a carrier solution prior to being made up in the desired concentration in filtered seawater (Allen, 1987). CB has come under the scrutiny of the United States Food and Drug Administration because of its potential danger to hatchery personnel (Eudeline *et al.*, 2000). The need for DMSO, and the highly toxic nature of CB make it less desirable as a useful portion of a commercial hatchery protocol.

The second chemical used to induce triploidy, 6-dimethylaminopurine (6-DMAP), is an analogue of puromycin. Puromycin is known to inhibit both protein synthesis and cleavage in marine eggs. The sensitive stage in egg development appears to be the formation of the mitotic apparatus as neither DNA synthesis nor the actual process of cell furrowing is affected by the inhibitor. It is recognized that 6-DMAP acts by inhibiting a relevant protein kinase activity in egg cleavage, acting on protein phosphorylation. Immunocytochemical studies using anti-tubulin antibodies have demonstrated that 6-DMAP treatments can disrupt the metaphase spindles in mouse oocytes (Rime *et al.*, 1989) and in sea urchin embryos (Néant *et al.*, 1989; Defresne *et al.*, 1991). Furthermore, electron microscopic analysis in mouse oocytes has confirmed that 6-DMAP can lead to disorganization of spindles and microtubule depolymerization (Szollosi *et al.*,

1991). These cellular effects explain how the triploidy state can be induced by blocking both chromosome movement and by blocking extrusion of polar bodies. The chromosomes are directly involved in the process, as opposed to CB where only the myofilaments are involved (Gérard *et al.*, 1999). Although 6-DMAP inhibits cleavage (polar body formation) at a minimum concentration 1 ½ to 3 times more than that of puromycin, it acts more rapidly. Rebhun *et al.* (1973) found that 6-DMAP inhibits cleavage if applied up to 5 minutes prior to the time of cleavage in clam eggs whereas similar effects are not seen for at least ½ hour after treatment by puromycin. This, along with the fact that 6-DMAP is relatively nontoxic and is easily dissolved in water, make 6-DMAP a well-suited candidate for triploid induction. More importantly, this water-soluble drug washes out of eggs easily, and the egg resumes normal development after treatment (Néant, 1988).

Heat shock has also been found to be useful in inducing triploidy. Cold and hydrostatic pressure can depolymerize *in vivo* mitotic microtubules (Inoué *et al.*, 1975). It is suggested that both hot and cold shocks affect cytoskeletal proteins in the case of triploid induction (Yamamoto and Sugawara, 1988). Yamamoto and Sugawara (1988) demonstrated that a hot shock, or increase in temperature, was more effective than a cold shock for producing triploid *Mytilus edulis*. Temperature shocks have often been used in conjunction with one of the two aforementioned chemicals with measurable success (Lutz, 1998). A 10°C increase in temperature throughout treatment of the fertilized eggs is effective in combination with other methods in the industry (Davis, pers. comm.). Hydrostatic pressure is the least effective when compared with other non-chemical induction methods. When considering its commercial limitations with regards to obtaining a pressure vessel large enough to induce sufficient quantities the use of a pressure shock is not considered commercially viable (Allen, 1987).

The important considerations for developing a triploid inducing technique are effectiveness and safety for commercial use. As 6-DMAP is far less dangerous than CB and equally effective in most cases (Desrosiers *et al.*, 1993), it was selected as the chemical induction agent for testing. In the case of triploid induction in the local blue mussel, *Mytilus edulis*, 6-DMAP and a heat shock of approximately 10°C were used as the parameters for triploid induction method development and optimization. For initial experimentation the tested 6-DMAP concentrations are similar to those used by Dèsrosiers *et al.* (1993) who ranged concentrations from 0 to 600 μmol/L. As the resolution in their results gave clear results in most cases, this same range was selected for experimentation in this case. It was also decided that both temperatures of 15°C and 20°C would be investigated as they are both common, and easily achievable working temperatures in shellfish hatcheries. 20°C was selected as it is a commonly used temperature in most shellfish hatcheries, and 15°C was selected as it is closer to the temperature during the natural local spawning in this species and also common in many shellfish hatcheries.

3.2 Materials and Methods

All experiments were conducted at the Ellerslie Shellfish Hatchery (ESH), in Ellerslie, PEI. Flow cytometry to define ploidy level in samples was performed at the University of Washington in Seattle, Washington, or at the Whiskey Creek Shellfish Hatchery in Tillamook, Oregon. The broodstock for all experiments were obtained from a mussel lease in vicinity of Lennox Island, PEI. In the case of each experiment they were conditioned using the system described in the Materials and Methods section of Chapter 2, section 2.2.1. The method of spawning in each case was identical to that described in the same section.

3.2.1 Experiment #1 – The Effect of 6-DMAP Concentration at Two Temperatures on Percent Triploid Induction

The sperm from six males was pooled for this experiment. This sperm was used to fertilize the pooled eggs from four females. Pooled eggs from the individually spawned mussels were counted and loaded into 1L plastic beakers. Eggs were loaded to a density of 100,000 eggs per beaker, or 100 eggs per ml of 1 μm filtered sea water (FSW). Eggs were then fertilized with enough sperm to account for approximately 5-25 active sperm per egg. A concentration of 5-25 sperm per egg is generally accepted as a suitable sperm concentration for optimal fertilization in industry.

Treatments in this experiment consisted of seven concentrations of 6-DMAP and a control at two temperatures. Each treatment had 3 replicates, giving a total of (7+1=8 chemical concentration treatments)(2 temperatures)(3 replicates per treatment) = 48 total replicates.

Eight concentrations of 6-DMAP used were 0 (control), 50, 100, 200, 300, 400, 500, and 600 μmol/L. 6-DMAP was added by calculating and weighing the proper amount to make up the correct concentration for all treatments. Each amount of 6-DMAP was then placed in a labeled aluminum foil packet. When a treatment was initiated the chemical was emptied into the beaker and rapidly mixed into the water until it was dissolved. Egg suspensions were mixed every few minutes throughout the treatment duration to ensure good chemical contact with all eggs for the treatment duration.

Temperatures of 15°C and 20°C were investigated. Although 15°C was shown to be less synchronous in experiment # 1 of chapter 2, these results were not yet obtained at the time of the current experiment. In both cases water baths were used to keep egg suspensions at the proper temperature. All beakers containing egg suspensions were allowed to sit for approximately 30 minutes prior to fertilization to ensure that eggs were sufficiently swollen to optimize

fertilization (Allen *et al.*, 1989). This also allowed the eggs to acclimate to the proper temperature just prior to fertilization. Sperm was added to each treatment and the time was noted for each. All treatments were staggered by 2 minute intervals. For example, the 100 μmol/L treatments were performed 2 minutes prior to the 200 μmol/L treatments. This was done in order to facilitate mixing and rinsing of the treatments in an orderly and time sensitive fashion.

All eggs were treated at 21 minutes post fertilization for a 20 minute duration. At the conclusion of the treatment duration the eggs (50-70μm) were thoroughly rinsed, placed onto a 20 μm sieve, and resuspended into the beaker with new FSW.

The egg suspensions were allowed to incubate for 24 hours and then samples were examined for both survivorship and ploidy. The survivorship sample involved collecting 10 ml of the suspension and fixing it by adding 5-10 drops of Lugol's iodine; these samples would be viewed later using light microscopy. In the remaining suspension, embryos that had grown to the D-stage were collected by rinsing them onto a 64 μm sieve. A Pasteur pipet was used to sample these from the sieve and place a 1mm plug of sampled embryos into a 15 ml centrifuge tube. These samples were then shipped live to the University of Washington for ploidy determination.

3.2.2 Experiment #2 – The Evaluation of Several Treatment Windows Suggested by The Described Meiotic Events

The sperm from five males was pooled for this experiment and was used to fertilize the pooled eggs from 12 females. This experiment was run in the same manner as experiment #1 with regards to the concentration of eggs and sperm used, the staggering of treatments time wise, the use of 6-DMAP, and egg suspension rinsing and sampling.

Treatment duration was a component of experiment #2. All treatments used a temperature of 20°C and a 6-DMAP concentration of 400 μmol/L. The treatments investigated included different treatment starting times based on the observed meiotic events in experiment #2 of chapter 2 (Figure 2.1). Also investigated were different treatment durations. Four treatment durations of 5, 10, 15, and 20 minutes were investigated along with 4 treatment starting times of 24, 27, 30, and 33 minutes post fertilization. There was an additional control treatment for comparison. Each treatment had 3 replicates, giving a total of (4 duration treatments)(4 treatment starting times)(3 replicates per treatment)+ 3 control replicates = 51 total replicates.

Samples were taken for both ploidy and survivorship in the same way as in experiment #1.

3.2.3 Experiment #3 – The Optimization of The 6-DMAP Concentration on Several Treatment Windows and The Effect of The Addition of a Heat Shock

The sperm from ten males was pooled for this experiment and was used to fertilize the pooled eggs from 15 females. This experiment was run in the same manner as experiment #1 with regards to the concentration of eggs and sperm used, the staggering of treatments time wise, the use of 6-DMAP, and egg suspension rinsing and sampling.

There were three combinations of treatment starting times and treatment durations investigated in this experiment. They were a treatment at 20 minutes post fertilization for 15 minutes, a treatment at 24 minutes post fertilization for 10 minutes, and a treatment at 30 minutes post fertilization for a duration of 5 minutes. As well, the effect of an addition of a 10°C heat shock (increase from 20 °C to 30°C) was investigated for each of these treatment combinations. These treatments used 6-DMAP concentrations of 0, 50, 100, 200, 300, and 400 μmol/L in order to reassess the effect of 6-DMAP concentration under these treatment parameters. The treatments

investigated included different treatment starting times based on the observations of experiment #2. Each treatment had 3 replicates, giving a total of (3 treatment starting time and duration combinations)(2 heat treatments – with or without a 10°C heat shock)(5 chemical concentrations)(3 replicates per treatment) + 12 controls = 102 total replicates. The 12 control replicates consist of 3 replicates of a 10°C heat shock for each treatment time combination without 6-DMAP (= 9 controls) as well as 3 replicates of a no heat shock and no 6-DMAP treatment.

Samples were taken for both ploidy and survivorship in the same way as in experiments #1 and #2.

3.3 Results

3.3.1 Experiment #1 – The Effect of 6-DMAP Concentration at Two Temperatures on Percent Triploid Induction

Figure 3.1 is a summary of the results. In all treatments the number of live D-larvae was too low to run each replicate for ploidy analysis. Therefore, all three replicates for each treatment were combined for ploidy determination by flow cytometry. Also, the survivorship samples degraded as the fixative was ineffective and samples were not properly fixed. Therefore, there were no available survivorship data for this experiment.

There were no triploids in the controls, however with the addition of 6-DMAP, all of the tested concentrations except the 50 μmol/L treatment at 15°C produced triploids. In all cases except the 500 μmol/L concentration, the 15°C treatments gave a lower percent triploid than did the 20°C treatments. For example, at a concentration of 400 μmol/L the 15°C treatment gave a triploid percentage of 67% versus 81% for the 20°C treatment. For both temperatures the curve tends to decrease in slope after the concentration of 300 μmol/L. There is little increase in the

percent triploids with any increase in 6-DMAP concentration used beyond 500 µmol/L. The observation of a low percentage of triploids (9.8%) in the 20°C 500 µmol/L sample is unexplained and assumed to be an outlier in the analysis, possibly due to an unexplained poor sample quality.

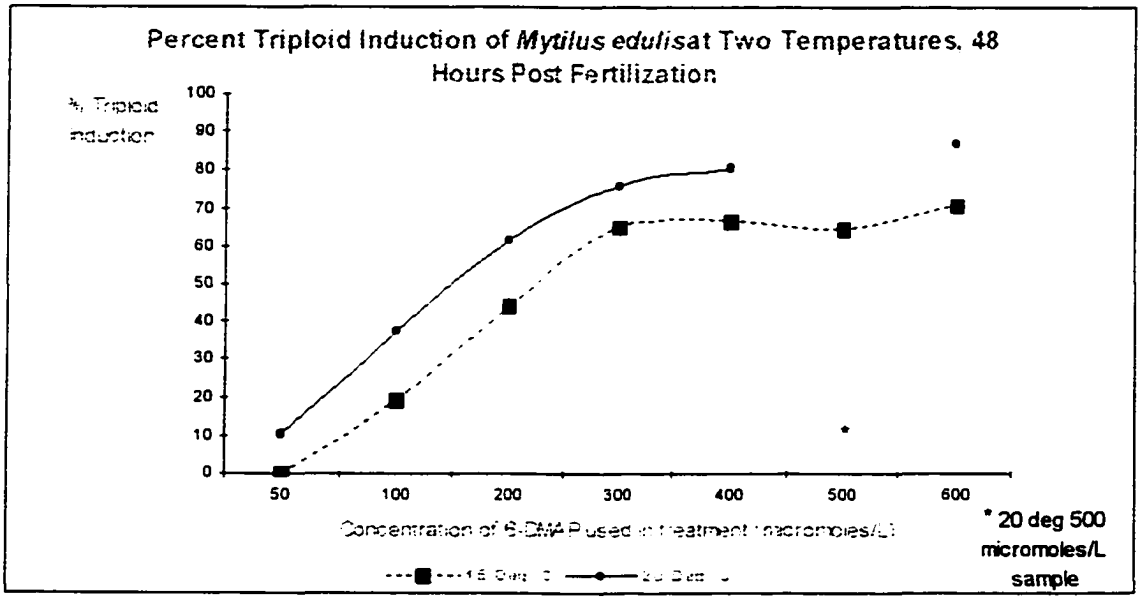

Figure 3.1: Percent triploid induction of *Mytilus edulis* at two temperatures using seven 6-DMAP concentrations.

3.3.2 Experiment #2 – The Evaluation of Several Treatment Windows Suggested by The Described Meiotic Events

The investigated treatment windows varied greatly in both the percentage of survivors to the D-stage (survivorship) and also in the percent triploid induced. Table III.I is a summary of the obtained results. The control samples tested showed a 37.6% survivorship to the D-stage, while there were no triploids present. The highest mean survivorship (9.33%) was found in the treatment begun 24 minutes post-fertilization for a duration of 5 minutes. The lowest mean survivorship (0%) was found in the treatment begun at 27 minutes post-fertilization for a duration of 20 minutes. The highest mean percent triploid induction (83.1%) was found in the

treatment begun 24 minutes post-fertilization for a duration of 10 minutes. The lowest mean percent triploid induction (0%) was found in the treatment begun at 33 minutes post-fertilization for a duration of 15 minutes

Tetraploid peaks were evident in the resulting printout from the flow cytometry obtained in a number of these treatments (see Table III.I). Therefore, some tetraploids were inadvertently induced in some of the treatments.

Table III.I: Survival and percent triploid induction summary for 16 treatment time windows in *Mytilus edulis.*

Temperature = 20°C Concentration of 6-DMAP = 400 µmol/L		Treatment Duration (minutes)				
		0	5	10	15	20
Treatment Time (minutes post fertilization)	0	*s = 37.60 ± 10.93 **t = 0				
	24		s = 9.33 ± 5.68 t = 56.1	s = 1.39 ± 1.59 t = 83.1	s = 0.98 ± 0.83 t = 59.3 ***TET	s = 0.28 ± 0.55 t = 68.6 TET
	27		s = 3.90 ± 2.76 t = 48.0	s = 0.84 ± 0.89 t = 65.8	s = 0.97 ± 1.04 t = 76.5 TET	s = 0 ± 0 t = 49.2 TET
	30		s = 6.96 ± 2.88 t = 51.8	s = 0.98 ± 1.51 t = 49.5	s = 3.2 ± 2.52 t = 11.6 TET	s = 0.42 ± 0.63 t = 17.2 TET
	33		s = 5.58 ± 2.09 t = 27.6	s = 2.23 ± 1.63 t = 14.9	s = 5.29 ± 2.99 t = 0	s = 3.07 ± 2.88 t = 2.2

*s = mean % survivorship to the D-veliger stage (mean of 3 replicates per treatment)
**t = mean % triploid of the sampled D-veligers
***TET = tetraploids were found in the ploidy sample for that treatment

3.3.3 Experiment #3 – The Optimization of The 6-DMAP Concentration on Several Treatment Windows and The Effect of The Addition of a Heat Shock

The ploidy samples for this experiment were not usable after shipping to the Whiskey Creek Shellfish Hatchery in Tillamook, Oregon. The experiment was performed twice, and both times no reliable ploidy data was obtained. Thus no useful data was obtained for this experiment.

3.4 Discussion

At the conclusion of this research the optimal procedure for induction of triploidy in *Mytilus edulis* was to treat eggs at 20°C with 400 µmol/L of 6-DMAP starting at 24 minutes post fertilization for a treatment time of 10 minutes. This treatment yielded 83.1% triploids in induced samples with a survivorship of 1.39% to the D- veliger stage (Table III.I).

Past attempts at inducing triploidy in *Mytilus edulis* have given a wide range of optimal treatments, resulting in a wide range of percent triploid induction and survivorship. Beaumont and Kelly (1989) produced, at best, 25% triploid-producing eggs using heat shock alone (from 15°C to 25°C at 10 minutes post-fertilization for 10 minutes). They measured percentage of eggs that were developing, and of those which percentage were developing normally. They found that, at best, approximately 10% of treated eggs developed. Of those that developed approximately 60% developed normally and would likely go on to develop as triploid embryos. One treatment had less than 5% development and less than 7% of those showed normal development. However, in the same study, they found that CB treatments could produce up to 60% triploid larvae in the optimal treatment group (using 1mg/L of CB at 5 minutes post-fertilization for 15 minutes). Again there was a significant reduction in the percentage that developed, and of those, which developed normally. Approximately 8% of treated eggs developed, and approximately 63% of those developed normally. The results from experiment #2 (Table III.I) illustrate some treatments

which showed better induction performance when compared to the aforementioned study, with up to 83.1% triploid induction (the 24-10 treatment) and up to 9.33% survivorship (the 24-5 treatment) to the D-veliger stage. Yamamoto and Sugawara (1988) were able to produce up to 97.4% triploid by the use of heat shock alone (from 20°C to 32°C at 20 minutes post-fertilization for 10 minutes), and found apparently no negative effect upon survival rates. These results have been questioned as they are much higher than those produced by Beaumont and Kelly (1989). Yamamoto and Sugawara (1988) report yields much higher than those found in the current experiments, which showed results slightly higher than those of Beaumont and Kelly (1989). Yamamoto and Sugawara (1988) gave high yields with heat shock alone despite the fact that 6-DMAP is generally accepted as being a more effective triploid inducer than temperature alone (Desrosiers *et al.*, 1993). Yamamoto and Sugawara also found 2-22% triploids in their controls that they attribute to handling stress. No other known triploid shellfish work has shown triploids in their controls. In the Chilean blue mussel, *Mytilus chilensis*, Toro and Sastre (1995) found an optimal induction treatment using heat alone (from 18°C to 32°C at 10 minutes post-fertilization for 10 minutes) to induce 51% triploid with 29% survival to an age of 15 days. The optimal percent triploid induction in their study was lower than in the current experiments, the survival rate was much higher however.

Although percent triploid induction is comparably high (over 50%) in some treatments for these experiments when compared with many shellfish studied (Beaumont and Fairbrother, 1991), survivorship is lower than expected. Future optimization of the triploid induction method should focus on increasing survivorship while not compromising percent triploid induction. Improved hatchery techniques and husbandry may also increase survivorship, but cannot help to increase the percent triploid. Possible further optimization using heat as an additional factor, as

was used without results in experiment #3, could also increase survivorship. This is evident as the previously mentioned studies using heat shock as an inducer showed higher suvivorship than did the current experiments. However, the optimal treatment obtained in the current study is the one yielding the highest percent triploid larvae. This is due to the specific concerns of the mussel industry.

The mussel industry is concerned that the triploidy procedure produces a high percent triploid and a high survivorship. It ultimately requires a method producing nearly 100% triploids. The main concern in PEI is that mussels maintain quality (while being shipped) during the spawning season. Ripe mussels (ready to spawn) can spawn during shipping due to mechanical or temperature stress (Gallant, pers. comm). If even a small percentage of a shipped crop were diploid and ripe, spawning may occur and consumer dissatisfaction could result. Therefore, the scientific approach has been to try and select a method that produces the highest percentage of triploids.

However, this has to be balanced with finding a method that produces a high survivorship of the triploids. The hatchery can place a great deal of effort into spawning the mussels and producing the triploids, particularly if they must condition the mussels out of season prior to spawning. Therefore, in order to make optimal use of resources, a method must be compromised so that it not only produces a high percentage of triploids, but it also produces a relatively high survivorship of the triploids. Taylor Resources, a hatchery that commercially produces triploid *Mytilus galloprovinciallis* in Washington, USA, typically obtains 95-100% triploid with 10-15% survivorship (Davis, pers. comm.).

Larval samples taken for ploidy and shipped live to the Whiskey Creek Shellfish Hatchery in Tillamook, Oregon were not useful for experiment # 3. Both times this experiment

was performed, upon arrival in Oregon the larval samples were analyzed and no ploidy determination could be made. Both times there were no peaks visible using flow cytometry, suggesting that there were no live larvae at the time of flow cytometry sample preparation (upon arrival after shipping). However, samples were checked prior to shipping and found to contain D-veligers. One possible explanation is that temperature changes or stress during shipping caused all larvae to die and begin DNA degradation. This never occurred in previous samples however. It was noted upon arrival that the samples contained a large amount of debris, suggesting that the water filtration system at the ESH may have been inadequate, and possibly attributed to fouling of the samples by allowing harmful bacteria and other contaminants into the samples.

3.4.1 Experiment #1 – The Effect of 6-DMAP Concentration at Two Temperatures on Percent Triploid Induction

As the samples for each treatment had to be pooled there was only 1 replicate per treatment. In all cases except the presumed poor sample at 500 μmol/L, the 20°C samples had a higher percentage of triploids than did the 15°C sample. The reason for this is that the eggs are more synchronous in terms of their meiotic stage, resulting in a higher percentage of eggs in the effective treatment stage and thus a higher percent triploid. This is in concordance with the results from experiment #1 in chapter 2 (section 2.3.1) which showed that there was a higher degree of meiotic synchrony at 20°C compared to 15°C. This is also supported in the literature as Lu (1986) found that meiotic events of the eggs are more synchronous at higher temperatures (not exceeding the physiological limits). In past studies, treatments with a higher degree of meiotic synchrony allow for more effective induction treatments as more eggs are in the target stage during treatment, resulting in a higher percent triploid and a higher survivorship (Downing

and Allen, 1987; Allen *et al.*, 1989; Gérard *et al.*, 1994). There was no survivorship data obtained from this experiment. This was likely due to the Lugol's iodine fixative being outdated. However, in light of these past studies and given that the 20°C gave both a higher percent triploid induction and a higher degree of meiotic synchrony (see section 2.3.1), the 20°C treatment is more optimal than the 15°C treatment. The optimal 6-DMAP concentration treatment window suggested by this experiment is between 300 and 500 μmol/L.

3.4.2 Experiment #2 – The Evaluation of Several Treatment Windows Suggested by The Described Meiotic Events

Survivorship was low in all cases for this experiment. This was likely because a relatively high concentration of 6-DMAP (400 μmol/L) was used. A decrease in the number of normally developed D-larvae (presumed to not be capable of survival) with an increase in 6-DMAP concentration was reported by Gérard *et al.* (1994). In this case the relatively high 6-DMAP concentration was required however, to ensure that a suitably high percentage of triploids were produced, and was based on the results from experiment #1 (Figure 3.1).

The results experiment #2 (Table III.I) were used to select the 3 induction test windows used in experiment #3. A treatment beginning at 24 minutes post fertilization for 10 minutes was selected for further examination in experiment #3 as it gave the highest percentage of triploids. During the optimization process it is useful to pick the best treatment timing from one experiment and use it, as well as one slightly higher and lower in future experiments. This helps give the optimization resolution so that at every step the experimenter knows that they are moving in the right direction with respect to each factor (e.g. increasing survivorship by decreasing 6-DMAP concentration). In order to bracket the induction timing a treatment not starting at 24 minutes post fertilization was selected. This was a starting time of 30 minutes post

fertilization for a duration of 5 minutes, giving a relatively high percent triploid induction (51.8%) and percent survivorship (6.96). In order to bracket lower for the next experiment a time lower than 24 minutes post fertilization was selected.

Figure 2.1 (chapter 2, section 2.3.2) illustrates that if a starting time of 20 minutes is used with a duration of 15 minutes this would act to effectively block meiosis at just previous to anaphase 2, slightly earlier than our current target of 50:50 anaphase 2:telophase 2. Although this is earlier than the stage suggested by Vadopalas (1999), it is suggested by Desrosiers *et al.* (1993) as being the optimal time to treat for 6-DMAP induction. In order to test whether this might be more optimal it was added as a treatment as well for further optimization in experiment #3. Experiment #3 did not yield results however, and this could not be examined.

Treatments initiated at 24 minutes post fertilization produced both the highest percent triploid (83.1 at a duration of 10 minutes) and the highest survivorship (9.33 at a duration of 5 minutes). As previously mentioned, Taylor Resources commercially produces triploid *Mytilus galloprovinciallis* and typically obtains 95-100% triploid with 10-15% survivorship (Davis, pers. comm.). It is difficult to compare these refined procedures with the results from these preliminary experiments, as much more optimization is required to reach commercial relevance. As these were preliminary experiments however, a triploid percentage of greater than 50% with as high a survivorship as possible was considered to be acceptable. This was based on the caveat that further optimization would improve both the percent triploid induction and survivorship. Difficulty in conditioning animals out of the natural spawning season, transportation, having to develop novel rearing and setting techniques, logistics, as well as the failed attempts at experiments #2 and #3 all made the anticipated further optimization impossible within the time constraints of this graduate program.

One further noteworthy result is the incidental production of tetraploid mussel larvae during some of the treatments in experiment #2 (Table III.I). Six different treatment combinations produced some measurable number of tetraploids. There is commercial significance in these findings. As mentioned in chapter 1 (section 1.2.6), triploids can be produced at a rate of 100% with a high survivorship without using chemicals by mating a tetraploid to a diploid. Therefore if tetraploids could successfully be produced and reared, they might become commercially important animals in the production of triploid mussels. Scarpa *et al.* (1993) found that tetraploidy could be induced in *Mytilus galloprovincialis* by suppression of both polar body 1 and 2. This might be the mechanism for the production of the tetraploids found in these results. However, when these tetraploid treatments are compared with Figure 2.1 in chapter 2, a pattern that might explain another mechanism becomes evident. All tetraploid producing treatments but the 24 minutes post fertilization for 15 minutes treatment overlap cell cleavage (Figure 2.1). Theoretically, if an inhibition treatment overlaps cell cleavage, the chromosomes will divide into two sets but the cell will not cleave. The result will be one cell with double the diploid number of chromosomes and subsequent mitotic divisions will result in a tetraploid larvae (Bearmont and Fairbrother, 1991). This has been shown as a viable method of tetraploid induction. Guo *et al.* (1994) attempted to produce tetraploid induction by inhibiting mitotic cell cleavage with heat shocks, producing up to 45% in one case, however no tetraploids in their study survived past the D-stage of development. Future work on triploid production of *Mytilus edulis* may focus on the production of tetraploid broodstock used to produce 100% triploid larvae without the use of chemicals. This is how many commercial triploid Pacific oysters are now produced (Chew, 2000).

3.4.3 Considerations for Future Optimization Efforts

Ploidy samples were lost or deemed unusable after or during shipping. Therefore, it is suggested that any future work on triploid induction in this species be conducted with a closer source of a flow cytometer and set up for ploidy determination. It would also be useful to have a flow cytometer to speed up the time between trials as sample processing was not rapid in all cases for this project. For the purposes of this project, the ploidy samples were sent away for processing as there was no immediate access to a flow cytometer. As well, its operation for this application without an experienced technician requires a certain level of expertise which was determined to be outside the confines of this graduate program. This should be a major consideration for any future work.

The current investigated methods for producing triploid *Mytilus edulis* are not yet optimized to commercial levels. However, it has been demonstrated that triploid mussels can be produced by using the puromycin analogue 6-DMAP at various concentrations. The findings described are a basis for further work in the optimization of this technique.

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4. A FIELD COMPARISON OF TRIPLOID AND DIPLOID *MYTILUS EDULIS*

4.1 Introduction

Various studies conducted since the advent of triploid molluscs in 1981 have shown their growth benefits and lack of reproductive capacity (Lutz, 1998). These qualities have been shown to be useful to the shellfish aquaculture industry. Yields have been increased and triploid product has been shown to be more marketable during the natural spawning season (Allen, 1988; Allen *et al.*, 1989; Baker, 1996; Lutz, 1998). One such case is that of the Pacifc oyster, *Crassostrea gigas*, which consumers have been shown to prefer when compared to diploids during the spawning season (Allen and Downing, 1991).

Mussels are dioecious, i.e., male or female (Mallet and Myrand, 1995). Gametogenesis occurs in numerous ducts (terminating in genital follicles) found in the mantle. Reproductive development begins slowly during the winter months and accelerates with the onset of spring phytoplankton blooms (Kautsky, 1982; Mallet and Myrand, 1995; Thompson, 1984). The mechanism and cues for spawning are poorly understood in mussels (Scarrat, 1993). In *Mytilus edulis* a rise in temperature above 10°C to 12°C is generally thought to be essential to spawning (Bayne, 1976; Mallet and Myrand, 1995). High food levels, a physical disturbance such as a storm, or water movement during spring tides are also believed to trigger spawning (Newell *et al.*, 1991; Bayne, 1976).

An understanding of the inability of triploids to spawn is key to the importance to the commercial value of triploid mussels (Chapter 1, section 1.4). The PEI mussel industry requires that triploid mussels do not spawn during the natural spawning season. An evaluation of the spawning or lack of spawning in triploids is most easily and accurately facilitated by evaluation of gametogenesis in both triploids and diploids during the spawning season. Histological

preparations provide the most exact assessment of the gametic state of the molluscan gonad. What is seen in the sections represents fairly closely the situation in the gonad as the state of development of individual cells can be seen (Quayle and Newkirk, 1989). In a female mussel the genital follicle may be referred to as the ovary. In the ovary oogonia are budded off from the germinal epithelium. These divide to form early oocytes. Early oocytes are connected by a broad stalk that gradually becomes more slender, eventually rupturing, leaving the mature ova free within the follicular cavity. In the male mussel the genital follicle may be referred to as the testis. In the testis, spermatogonia are budded off from the germinal epithelium. The spermatogonia give rise to concentric bands of spermatocytes. The spermatocytes undergo meiosis to give rise to spermatids, which become spermatozoa with developed tails, converging towards the center of the follicle in the form of dense lamellae (Lowe *et al.*, 1982; Gossling, 1992). Figure 1.1 (Chapter 1) provides an illustration of spermatogenesis and oogenesis.

There are numerous studies that tout the merits of triploid bivalve molluscs for their improved growth and their lack of reproductive output when compared to diploids (Guo, 2001). Improved growth makes them suitable for culture by increasing production and profits (Stanley *et al.*, 1984; Allen *et al.*, 1986; Allen *et al.* 1989; Beaumont and Fairbrother, 1991, Lutz, 1998). The lack of reproduction during the natural spawning season makes them more marketable than their diploid counterparts during the peak of the local spawning season (Allen and Downing, 1991). This sterility also makes them candidates for the use of exotic or introduced species, where there is a reduced chance of them interbreeding or displacing naturally occurring species (Allen, 1987; Guo *et al.*, 2001).

To investigate the possibility of a growth advantage of triploids when compared to diploids both shell length, and a measure of the condition index of the animals is used. Greatest

shell length (GSL), is the most used measurement of growth in bivalves and should be included in any growth study of bivalves (Quayle and Newkirk, 1989). Shell growth rates are of commercial importance as mussels are generally harvested, and sorted based on shell length. Therefore, higher growth rates mean faster commercial production. There are various condition indices (CI's), or condition factors. all of which can be used to indicate the relative amount of the internal shell volume that is occupied by the body (Crosby and Gale, 1990). This can be indicated by a relative measure of either the shell volume or the shell weight to the tissue weight (Quayle and Newkirk, 1989). The degree to which a shell is full is an indication of the relative condition and commercial value of the animal. Less full animals are of less commercial value than more full ones, making this measure specifically important to the question of the commercial importance of triploid mussels.

The objective of this study was to evaluate the field performance of triploid mussels when compared to a diploid control. This involves two main objectives:

1) to evaluate the differences in the growth of triploids and diploids in various growout sites using measures of (CI), greatest shell length (GSL), and dry tissue;

2) to evaluate, based on gametogenesis rating observations, the ability of triploids to spawn when compared to diploids during the natural spawning periods at various sites.

4.2 Materials and Methods

4.2.1 Production and Rearing of The Experimental Animals

Triploid mussels were produced during one of several spawns for use in the field performance trials. All hatchery work was performed at the Ellerslie Shellfish Hatchery in Ellerslie (ESH), Prince Edward Island (PEI). During each spawn, one half of the fertilized eggs

were treated, producing triploids, and one half of the eggs were not treated, producing diploids. Both triploid and diploid animals had the same parents. The triploid group will be referred to as the experimental group, and the diploid group will be referred to as the control group. The percent triploidy was determined using flow cytometry at either the University of Washington or the Whiskey Creek Shellfish Hatchery. The animals used in all field trials came from a spawn performed on May 16, 1999. Broodstock was collected from a mussel lease in the Lennox Island area of PEI. As the animals were in spawning condition upon arrival at the hatchery, they were induced to spawn upon arrival using the methods described in chapter 2, section 2.2.1. The eggs from 24 females and the sperm from 6 males was pooled for use. Approximately 75 million eggs were obtained from the females. These were divided equally into 2 groups. The control group was fertilized from the pooled sperm and allowed to develop into diploid controls. The experimental group was also fertilized from the pooled sperm and then treated to produce triploids. Triploidy was induced using a treatment of 400 μmol/L of 6-DMAP for a 20 minute treatment at 21 minutes post-fertilization. Fertilization and treatment of the eggs was facilitated in a clean 20 L bucket filled with filtered 1 μm filtered sea water (FSW). As with previous triploid mussel work, care was taken to not contaminate eggs prematurely with sperm, and all lab supplies were rinsed in a 5% bleach solution and then in fresh water before and after use. Each set of embryos were transferred to 450L conical larvae tanks for subsequent rearing. Larval water changes were performed every 2 days using the procedures standard at the ESH. As the larvae grew they were divided into more tanks so as to maximize yield by decreasing stocking density. Larval counts and quality were assessed during water changes. Larvae were fed a mix of microalgae, consisting of both flagellates and diatoms commonly grown at the hatchery for bivalve larvae.

Frayed rope previously used in the lobster fishery, the industry norm for wild spat collection, was used to set the animals. Frayed rope pieces (with a 30 cm metal spike acting as a weight attached on the bottom) was suspended in each of the tanks. This rope was soaked for approximately 1 week in filtered sea water (FSW) prior to use. This process, known as "seasoning" is known to allow better spat attachment with regards to wild spat collection. After 21 days animals began to set and attach to the tank walls and collector ropes. Bivalves that have set and metamorphosed into sessile animals are referred to as spat. After an additional 10 days only abnormal larvae (swimming abnormally, or showing developmental abnormalities) were left in suspension. These were discarded, and care was focused now solely on the spat attached to the collectors.

The spat were fed a dietary supplement to improve growth and to reduce the need for cultured microalgae, which is costly and time consuming to grow. This supplement, Dacosa Gold ™, is a freeze dried heterotrophic algae. It has been shown to be effective in the replacement of live algae in *Mytilus galloprovincialis* and is known to be high in lipids essential to good larval nutrition (Langdon and Önal, 1999). Each day every tank received 20g of this substance blended in 1L of water for 8 minutes as per the label instructions.

On August 1, 1999 (77 days after being spawned) approximately half of the control and experimental spat were deployed directly on mussel leases on the hatchery rope collectors. The other half of the spat were retained and grown in a downwelling setup typically used at the hatchery. The deployment was done by attaching the collector ropes (used for setting in the hatchery) to mussel culture longlines in each of the various sites. Control collectors were clearly distinguished from the experimental ones by marking both types with different coloured string. These collectors were divided equally and distributed between all test sites. When deployed,

experimental and control collectors were alternately hung on the longlines. Each participating grower was required to harvest the collectors and sock both the control and experimental mussels separately in the fall of 1999 as is done in the local mussel industry. The resulting units were a series of experimental and control mussel socks at each site.

Juvenile mussels retained in the hatchery were grown in a downwelling system in which the water of the tank was forced up through a pipe using an airlift, and down over the animals which were held in small silos. The animals were kept off bottom by 243 μm screens, through which the water returned to the tank. In this way the mussel juveniles were constantly exposed to aerated water and a food supply. These animals were held in a static tank of FSW with algae and Dacosa Gold ™. Every 2 days these tanks were cleaned and both clean FSW and an algae/Dacosa Gold mixture were reintroduced to each tank.

On September 1, 1999 (108 days after being spawned) the remaining experimental and control spat were sieved on a 2 mm screen, and animals retained on the screen were counted. Approximately 5000 individuals from each group (experimental and control) were placed into separate 2 mm mesh spat bags measuring 60 cm in length and 20 cm in width. These bags were placed into modified lobster trap wire cages. Each cage held one experimental and one control bag. These cages were anchored to the bottom by concrete blocks. Floats were attached to the cage to keep the cages approximately 1 m above the sea floor. Experimental and control bags were again marked by different colours so as to enable easy distinguishing for sampling. Two cage units were placed at each of the three test sites. Participating growers placed 1 cage in that portion of their lease in which growth was highest, and the other in that portion of their lease in which growth was lowest. The cages were cleaned as required at each of the sites so as to reduce fouling of gear and maximize growth and survival.

4.2.2 Determination of The Experimental Sites

Animals were deployed in 3 sites with differing average mussel growth rates. The three test growout sites were selected in conjunction with the PEI Aquaculture Alliance and experienced mussel producers. The criteria for site selection included the capacity for grower participation, and the required growth rate. The objective was to identify any relationships between site quality (based on relative historical mussel growth) and triploid versus diploid performance. Given this goal, sites with historically low, average, and above average mussel growth compared with the PEI average were selected. The selected sites were:

1) St. Peter's Bay - below average mussel growth rate

2) Tracadie Bay - average mussel growth rate

3) Lennox Island (mussel lease in Lennox channel) – above average mussel growth rate.

4.2.3 Growth Measurements

For each sample, a random sample was obtained from either the collector or the cage unit. When sampling socks a separate sock was sampled each time. Mussels were sampled from approximately 1 m depth on the sock each time. Sampling from the cage units was done by randomly selecting a sample from each mesh bag. During each sampling, a large sample of mussels were obtained in the field. Samples were taken back to the laboratory where they were randomly selected again (to achieve the target sample size) and measured. Samples taken in the fall of 1999 were measured for greatest shell length (GSL) using Vernier calipers. Samples taken after the fall of 1999 included dry shell, dry tissue, and total dry weight for a condition index (CI), as well as GSL.

The following defines all measurements taken:

1) The greatest shell length (GSL) is defined as the greatest length mesurable from the umbo of the shell (anterior end) to the posterior end of the shell. All values reported are in millimeters.

2) The condition index (CI) is defined as: CI = (dry tissue wt (g) x 1000) /dry shell wt (g). This condition index is discussed by Crosby and Gale (1990), and was first introduced by Walne and Mann (1975) for use in comparisons with *Ostrea edulis* and *Crassostrea gigas*. Sample tissue and shell were dried in a drying oven at 60°C for 48 hours.

3) Ploidy determination was done by taking a small (1 mm) square section of gill tissue and fixing it in 4',6'-Diamidino-2-phenylindole Dihydrochlorate (DAPI)/detergent/DMSO solution (chapter 1, section 1.2.5). These samples were then shipped for ploidy determination by flow cytometry at the Whiskey Creek Shellfish Hatchery in Tillamook, Oregon. Each individual mussel measured was known to be either a diploid or a triploid.

4.2.4 Statistical Methods

All reported p-values were generated in the statistical analysis by use of the t-test statistic in order to determine whether or not tested means are equal. Reported p-values were generated comparing the means, and not the percent difference in the means. Assumptions of normality of the data as required for parametric tests were made in the cases where the t-test was used.

4.2.5 Gametogenesis Rating – Histological Samples

Histological samples were taken to rate gametogenesis in both diploids and triploids in order to determine whether there is a difference between the two in ability to spawn. For each

mussel tested a piece of the gonad was fixed in Davidson's solution. Each sample was imbedded in parafin and stained using a hematoxylin and eosin stain. The prepared slides were observed under light microscopy for both sex determination and gonadal cycle. The rating system used for gonadal cycle is a 5 rank description suggested by Ropes and Stickney (1965). This system of rating gametogenesis, though developed for *Mya arenaria*, is readily applicable to most bivalves and simple in its description. A simplified summary is located in Table IV.I.

Table IV.I: Gonadal cycle rating system used in this study, after Ropes and Stickney (1965).

Stage	Observable Characteristics	
	Male	Female
1 – Inactive	- sperm at the periphery of gonoduct with few if any sperm in the gonoduct center - some multinucleated dividing cells	- small, immature eggs evident in the gonoduct periphery - lipid droplets evident in the gonoduct -nutritive inclusions evident in the gonoduct
2 – Active	- sperm located throughout the gonoduct; some sperm may have tails observable late in this stage - some multinucleated dividing cells	- eggs beginning to grow from the gonoduct periphery to the center with stalks holding eggs to gonoduct wall enlarging -nutritive inclusions still evident in the gonoduct
3 – Ripe	- entire gonoduct filled with spermatozoa (ripe sperm); sperm tails evident and pointing towards center of gonoduct	- eggs near the center of the gonoduct and appearing larger with transparent nucleus enclosing a small opaque nucleolus evident
4 – Partially Spent	- few sperm left; remaining sperm only occupy the center of the gonoduct with very few sperm near the periphery - vacuolated cells evident in the gonoduct - pycnotic cells (necrotic cells) evident	- few stalked (mature) eggs left in the gonoducts -nutritive inclusions again becoming evident in the gonoduct
5 – Spent	- many vacuolated cells evident in the gonoduct - few if any sperm left in the gonoduct center and no sperm tails are evident - pycnotic cells (necrotic cells) evident - also evident are some non-pycnotic multinucleated dividing cells	- small, immature eggs evident in the gonoduct periphery - lipid droplets evident in the gonoduct -nutritive inclusions evident in the gonoduct - showing fewer lipid droplets and nutritive inclusions than active stage

4.2.6 Sampling Schedule

Growth comparisons between triploid and diploid controls were assessed at various times at the different test sites. At the St. Peter's Bay site there were two sampling dates. The first was in July of 2000. The sample consisted of 100 mussels from an experimental sock and 100 from a control sock. These mussels were measured for dry weights, CI, GSL, and ploidy. The second sampling date was in July of 2001 and consisted of 100 mussels from the experimental sock only. These mussels were measured for dry weights, CI, GSL, and ploidy. 100 mussels were also sampled for histology, GSL and ploidy.

At the Lennox Island site there were three separate sampling dates. The first sample was taken in November of 1999 when the mussels were transferred from collectors to socks. A sample of 200 mussels from both the experimental and control groups were measured for GSL and ploidy. The second sampling period, May of 2000, consisted of a growth (dry weights, CI, GSL) sample and a histology (to rate gametogenesis) sample. The growth sample included 100 mussels from the experimental group and 100 mussels from the control group. These were measured for dry weights, CI, GSL, and ploidy. The histology sample included 50 mussels taken from an experimental sock, and were measured for GSL and ploidy. A histology section was also taken from each mussel. The third sampling period was July of 2000 and also consisted of a growth sample and a histology sample. The growth sample consisted of 100 mussels from the experimental group and 100 mussels from the control group. The histology sample consisted of 100 mussels taken from an experimental sock and 100 mussels from a control sock. For both samples at this date measurements taken were the same as those for the May 2000 sampling date.

4.3 Results

Survivorship of the larvae one week after the spawn (May 22, 1999), based on eggs initially fertilized, was 24.3 % in the control and 0.45% for the experimental group. An initial % triploid evaluation at that time using flow cytometry showed that there were no triploids in the control group and there were approximately 83% triploid, and 17% diploid in the experimental group. On September 1, 1999 another ploidy sample determined that there were no triploids in the control group and the experimental group contained 80% triploid, and 20% diploid animals.

4.3.1 Growth Trials

Prior to cage deployment on September 1, 1999 the experimental group mean GSL was 5.07±1.39mm (n=100). The control group mean GSL was 3.57±1.02mm (n=100). As this was not a direct comparison and there was no controlled experiment done on growth in the hatchery as stocking densities and feeding rates differed, statistical comparison would be meaningless.

On October 29, 1999 100 randomly selected mussels from both the control and the experimental group were taken from the cage units at each test site. Measurements were taken and individuals were sampled for ploidy determination. At that time triploids were found to contaminate (up to 50%) the controls in all three test sites. This caused a lack of confidence in measurements for both the present and future with regards to the cages. As these were increasingly difficult to maintain and the value of data obtained from them could be questioned given the contamination of groups, the cages were excluded from further analysis.

4.3.1.1 St. Peter's Bay

Results of the July of 2000 (11 months after being deployed) sampling are outlined in Table IV.II and Figure 4.1. There was a statistical difference in GSL between diploids on the control sock and diploids on the experimental sock ($p<0.001$). Both diploids and triploids are found on the experimental socks. This test was done to see if diploids from the experimental sock were similar to those on the control sock so that they could be pooled for further analysis. A larger pool of controls would allow for more statistical power to detect smaller differences between triploids and diploids. Thus, as the two groups of diploids were different, the diploids could not be pooled for statistical analysis in order to increase statisitical power. Therefore, the reported sample is taken from the triploid sock only. Within the experimental sock sample there were 10% triploid and 90% diploid. There were over 80% triploid and less than 20% diploid on the experimental collectors when deployed 11 months prior. There were no statistically significant differences in any of the measured characteristics between diploid controls and triploids sampled. Triploids showed a 1.05% larger shell length (GSL), but diploids showed a 4.43% greater CI (indicated by a negative number in Table IV.II).

Results of the July of 2001 sampling are outlined in Table IV.III and Figure 4.1. Four mussel ploidy samples were lost. These mussels were excluded from the analysis. There was 28% triploid in the experimental sock. Triploids showed statistically significant greater size and weight in all measures and showed a larger CI ($p<0.001$; Table IV.III). Mean triploid GSL was 10.95% larger than diploid controls, and triploids contained 62.82% more dry tissue. Triploids also had a 30.62% higher CI than diploids.

Table IV.II: A summary of the growth comparisons between diploid and triploid mussels at St. Peter's Bay test site in July of 2000.

Sample July 2000: n = 100; nt = 10 (# of triploids), nd = 90 (# of diploids); % triploid = 10%					
GSL in diploids from control sock and experimental sock were statistically different (p<0.001), Therefore, the diploid sock measurements were excluded					
	Length (mm)	Dry shell (g)	Dry Tissue (g)	Total dry (g)	Condition index
Number	nt=10; nd=90	nt=10; nd=90	nt=10; nd=90	nt=10; nd=90	nt=10; nd=90
Total mean	42.47	2.31	0.34	2.66	149.60
Triploid mean	42.87	2.48	0.36	2.84	143.70
Diploid mean	42.42	2.29	0.34	2.64	150.30
Difference in mean	0.45	0.19	0.02	0.21	-6.60
% Difference in mean	1.05	8.15	5.03	7.74	-4.43
p-value	0.48	0.12	0.32	0.14	0.24

Table IV.III: A summary of the growth comparisons between diploid and triploid mussels at St. Peter's Bay test site in July of 2001.

Sample June 2001: n = 96; nt = 27 (# of triploids), nd = 69 (# of diploids); % triploid = 28%					
	Length (mm)	Dry shell (g)	Dry Tissue (g)	Total dry (g)	Condition index
Number	t=27; d=69	t=27; d=69	t=27; d=69	T=27; d=69	t=27; d=69
Total mean	62.03	7.76	1.46	9.22	184.60
Triploid mean	66.91	9.37	2.12	11.49	225.30
Diploid mean	60.12	7.13	1.20	8.34	168.70
Difference in mean	6.79	2.23	0.92	3.15	56.50
% Difference in mean	10.95	28.77	62.82	34.16	30.62
p-value	<0.001	<0.001	<0.001	<0.001	<0.001

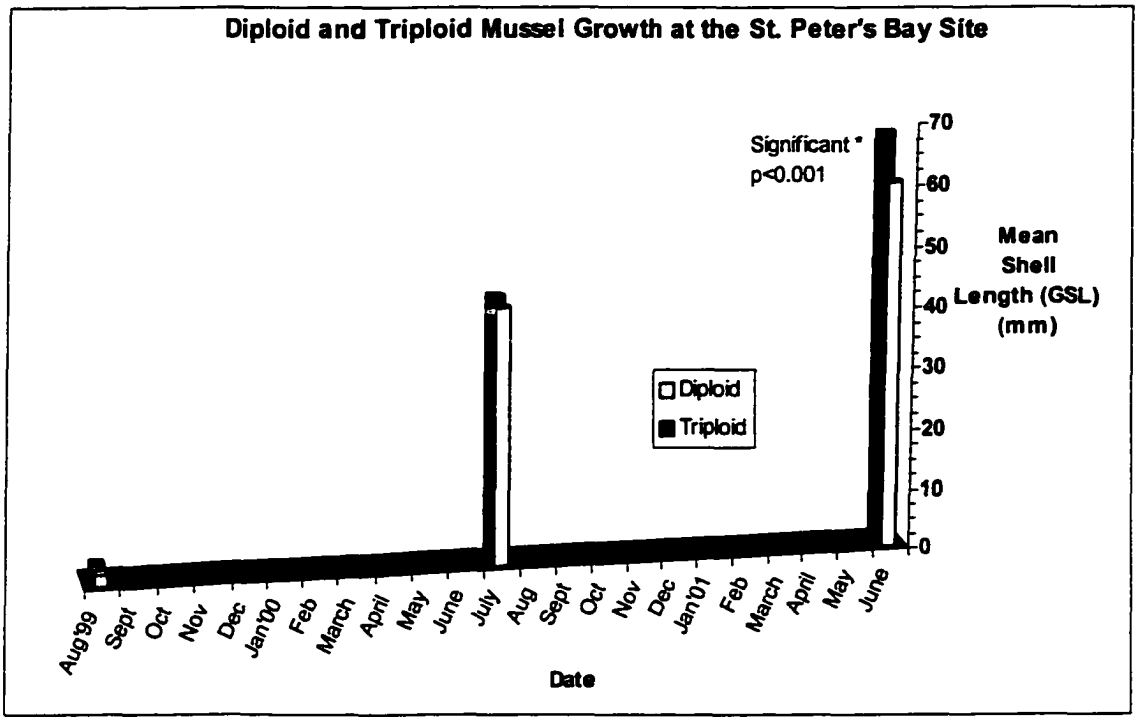

Figure 4.1: Growth of triploid and diploid mussels at the St. Peter's Bay test site.

4.3.1.2 Tracadie Bay

The deployed collectors were misplaced by the participating grower and therefore excluded from the study.

4.3.1.3 Lennox Island

Results of the November 1999 sampling (3 months after being deployed) are summarized in Table IV.IV. 100% of the sampled mussels in the experimental group were triploids. There was no statistical difference ($p>0.05$) in GSL between diploid and triploid mussels on socks (Table IV.IV and Figure 4.2).

Results of the May 2000 sampling (9 months after being deployed) are summarized in Table IV.V and Figure 4.2. Of the 100 mussels sampled from the experimental sock for GSL

and CI, 86% were triploid and 14% were diploid. Of the 50 mussels taken from the experimental sock for histology, 90% were triploid, and 10% were diploid. There was no statistical difference in GSL between triploids in both samples ($p=0.18$), therefore the samples were pooled for statistical analysis of GSL only. In all measures, triploids were significantly different from diploids, having a higher CI when compared with diploids (Table IV.V). Triploids showed a 10.05% larger shell length (GSL), a 24.23% greater dry tissue weight, and a 15.48% larger CI.

Results of the July 2000 sampling (11 months after being deployed) are summarized in Table IV.VI, Table IV.VII, and Figure 4.2. In the growth sample taken there was 91% triploid in the experimental group. Diploids in the experimental and control groups did not differ significantly in GSL ($p=0.08$) therefore diploids were pooled for statistical analysis (Table IV.VI). Triploids had a significantly larger GSL (8.09%, $p<0.001$). Dry tissue weight was 45.66% greater for triploids. Triploids also showed a 21.28% greater CI. Table IV.VII summarizes the results from the histology sample taken at this sampling period. There was a significant difference in GSL between respective diploids ($p=0.01$) and respective triploids ($p=0.02$) for GSL when the growth and histology samples were compared. Therefore the histology sample was analyzed separately. There were 90% triploids in the experimental group of the histology sample. The diploids in the control and experimental groups did not differ significantly in GSL ($p=0.17$), therefore they were pooled for the statistical analysis. Triploids had a 3.53% larger GSL than did diploids. This difference was statistically significant ($p<0.001$).

Table IV.IV: A summary of the growth comparisons between diploid and triploid mussels at the Lennox Island test site in November of 1999.

Sample November 1999: n = 400; nt (# triploid) = 200, nd (# diploid) = 200; % triploid = 100%	
	Length (mm)
Number	nt=200; nd=200
Total mean	34.54
Triploid mean	34.65
Diploid mean	34.43
Difference in mean	0.22
% Difference in mean	0.06
p-value	>0.05

Table IV.V: A summary of the growth comparisons between diploid and triploid mussels at the Lennox Island test site in May of 2000.

Sample May 2000: n = 150; nt (# triploid) = 131; nd (# diploid) = 19; % triploid = 87%					
	Length (mm)	Dry shell (g)	Dry Tissue (g)	Total dry (g)	Condition index
Number	nt=131; nd=19	nt=86; nd=14	nt=86; nd=14	nt=86; nd=14	nt=86; nd=14
Total mean	41.58	2.14	0.90	3.04	436.40
Trip mean	42.11	2.18	0.93	3.11	445.90
Dip mean	37.93	1.89	0.71	2.60	378.30
Difference in mean	4.18	0.29	0.22	0.51	67.60
% Difference in mean	10.05	13.69	24.23	16.81	15.48
p-value	<0.001	<0.001	<0.001	<0.001	0.045

Table IV.VI: A summary of the growth comparisons between diploid and triploid mussels at the Lennox Island test site in July of 2000.

Sample July 2000: n = 200; nt (# triploid) = 91; nd (# diploid) = 109; % triploid = 55% (combined); 91% triploid in the triploid group					
**GSL in diploids from control sock and experimental sock were not statistically different (p=0.18), therefore, the control and experimental diploids were pooled for analysis*					
	Length (mm)	Dry shell (g)	Dry Tissue (g)	Total dry (g)	Condition index
Number	nt=91;nd=109	nt=91; nd=109	nt=91; nd=109	nt=91; nd=109	nt=91; nd=109
Total mean	51.46	4.91	0.79	5.70	159.10
Trip mean	53.73	5.62	0.99	6.61	177.60
Dip mean	49.57	4.31	0.63	4.94	143.70
Difference in mean	4.16	1.30	0.36	1.67	33.90
% Difference in mean	8.09	26.56	45.66	29.21	21.28
p-value	<0.001	<0.001	<0.001	<0.001	<0.001

Table IV.VII: A summary of the GSL comparison in the histology sample between diploid and triploid mussels at the Lennox Island test site in July of 2000.

Sample July 2000: n = 200; nt (# triploid) = 90; nd (# diploid) = 110; % triploid = 90	
**There was a significant difference in GSL between respective diploids (p=0.01) and respective triploids (p=0.02) when the histology sample is compared to the growth sample **GSL in diploids from control sock and experimental sock were not statistically different (p=0.17)*	
	Length (mm)
Number	nt=90; nd=110
Total mean	51.80
Trip mean	52.81
Dip mean	50.98
Difference in mean	1.83
% Difference in mean	3.53
p-value	<0.001

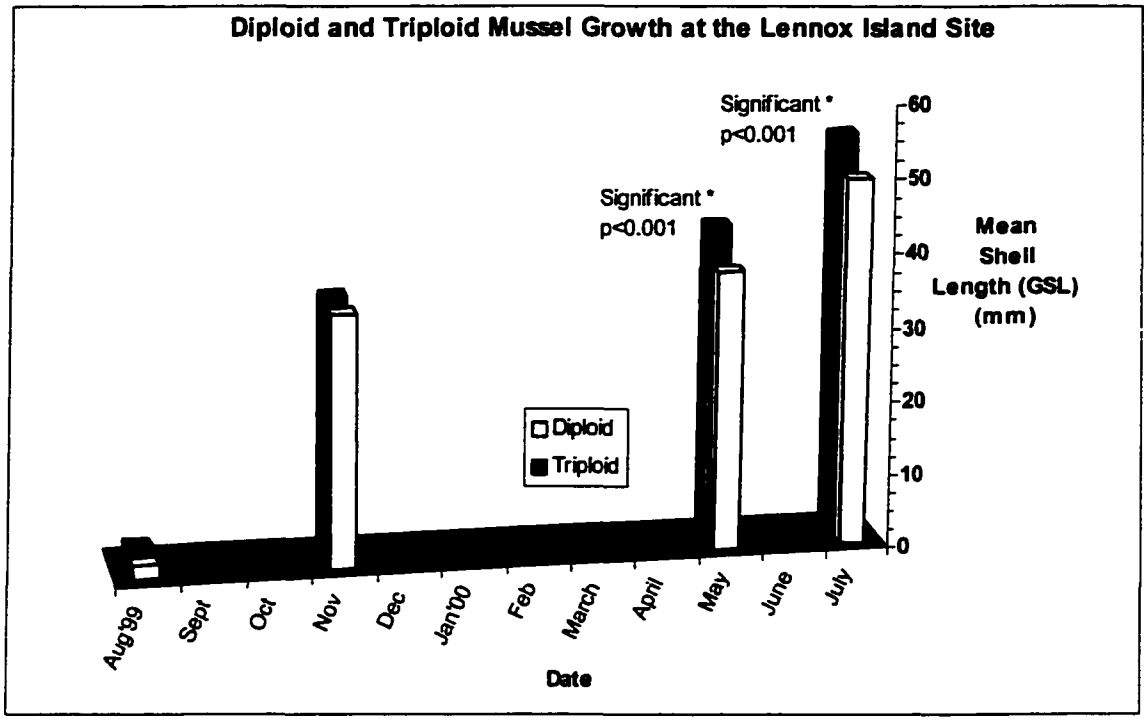

Figure 4.2: Growth of triploid and diploid mussels at the Lennox Island test site.

4.3.2 Gametogenesis Rating – Histological Samples

The Lennox Island sample taken on May 1, 2000 consisted of 50 mussels randomly sampled from an experimental sock. It was determined that there were 45 triploids and 5 diploids. Of the 45 triploids 44 were found to be male, and all except the 1 putative female were found to be in the active stage (not yet ripe, Table IV.I). All triploid males exhibited many spermatocytes in the gonads, but no mature spermatozoa. The female was scored as being in the spent stage. This was the closest category to describe the histology, although it did not appear as a normal spent female. Of the 5 diploids, 4 were female and 1 was male. All diploids were found to be in the ripe stage.

Figures 4.3 and 4.4 are photomicrographs of diploid and triploid male gonads observed during this sampling. Of note is the clear morphological difference between the two. At a lower

magnification (Figure 4.3) the retardation of gametogenesis is evident in the triploid by the evidence of vacuolated spaces within the follicle. At higher magnification (Figure 4.4) spermatocytes, giving rise to spermatids and spermatozoa (in the center of the follicle) are visible in the diploid follicle. By contrast the triploid follicle contains only spermatocytes, and shows no formation of spermatids or spermatozoa (which would be formed after a meiotic division, see section 1.2.2). Figures 4.5 and 4.6 are photomicrographs of an observed ripe female in comparison to the observed putative female triploid. At lower magnification (Figure 4.5) the evidence of gametogenesis retardation is evident in the triploid. The diploid contained many full follicles with some stalked oocytes and some free oocytes while the triploid showed virtually no follicle with severely degenerated germ tissue, and only scant malformed oocytes. At higher magnification (Figure 4.6) one can see clear nuclei in the diploid oocytes within the follicles while the follicle itself shows a large infiltration by hemocytes, with little identifiable germ tissue. Follicles and oocytes were difficult to find anywhere in this triploid gonad section.

In the Lennox Island sample taken on July 24, 2000 there were 90 triploids and 10 diploids (= 90% triploid) in the 100 mussels taken from the experimental group. These 10 diploids were pooled with the 100 from the diploid sample to give a pooled sample of 90 triploids and 110 diploids for analysis. Tables IV.VIII and IV.IX summarize the observations of sex and gonadal cycle for the respective diploids and triploids sampled. In the diploids 39% were females and 61% were males. Only 1 female (1% of the total) was found in the triploids. In the diploids 29% of mussels were still in the ripe stage while the remaining 71% had spawned, being either partially or completely spent. In the triploids there was no evidence of spawning. The one putative female showed severe retardation of gametogenesis (again scored as being in the spent stage) and the remaining 99% of triploids were in the active stage or in the inactive stage. Again,

there were only spermatocytes, and no mature spermatozoa evident in the testis of triploids. No triploids were in the ripe stage.

The ploidy samples from the St. Peter's Bay site taken in June of 2001 were inconclusive. The flow cytometry results showed various peaks as different ranges for these samples. In some cases there were as many as 6 ploidy peaks for one animal. The reason for the multiple peaks in unknown but may be related to DNA degradation of the samples.

Table IV.VIII: The sex and gonadal cycle distribution of diploid mussels sampled from the Lennox Island test site in July of 2000.

Diploid Histology Samples				
Gonadal Cycle Stage	**# of Females**	**# of Males**	**Total**	**% of Total**
1 – Inactive	0	0	**0**	**0**
2 – Active	0	0	**0**	**0**
3 – Ripe	14	18	**32**	**29**
4 – Partially Spent	12	20	**32**	**29**
5 – Spent	17	29	**46**	**42**
Total	**43**	**67**	**110**	
% of Total	**39**	**61**		

Table IV.IX: The sex and gonadal cycle distribution of triploid mussels sampled from the Lennox Island test site in July of 2000.

Triploid Histology Samples				
Gonadal Cycle Stage	**# of Females**	**# of Males**	**Total**	**% of Total**
1 – Inactive	0	42	**42**	**47**
2 – Active	0	47	**47**	**52**
3 – Ripe	0	0	**0**	**0**
4 – Partially Spent	0	0	**0**	**0**
5 – Spent	1	0	**1**	**1**
Total	**1**	**89**	**90**	
% of Total	**1**	**99**		

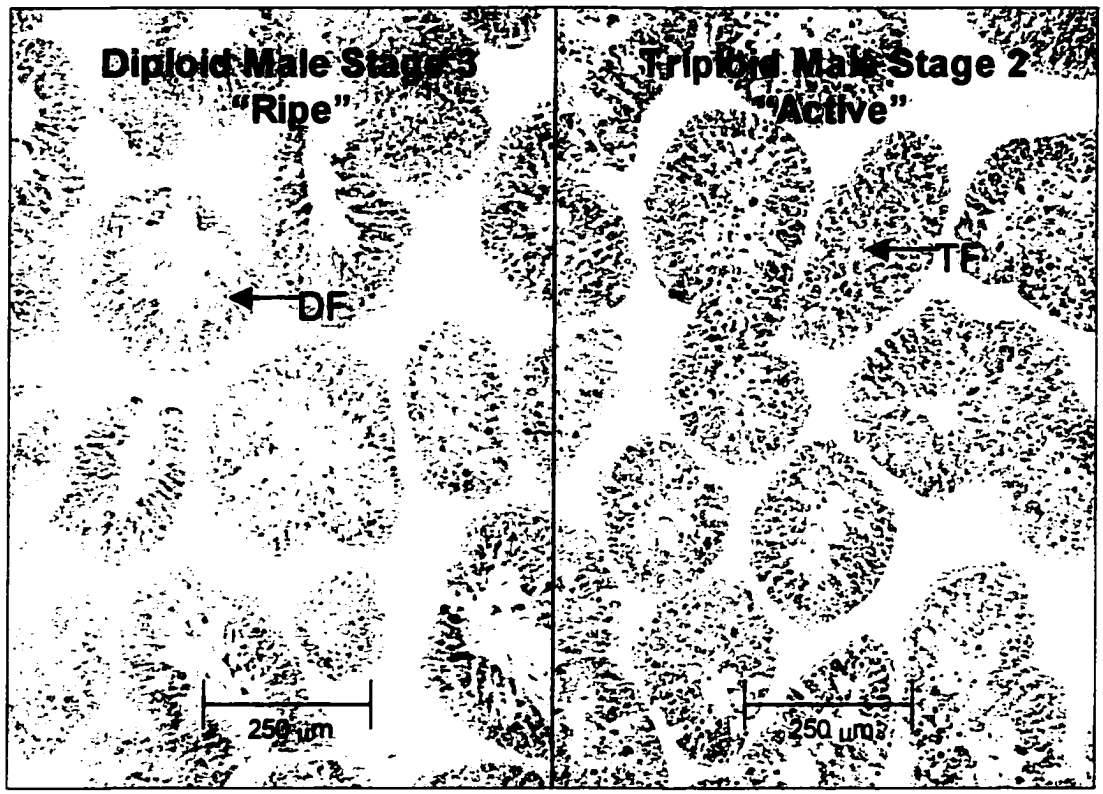

Figure 4.3: Photomicrographs of *Mytilus edulis* male gonad; 100X; H&E stain; diploid and triploid sampled from Lennox Island site May 1, 2000; DF = diploid follicle; TF = triploid follicle.

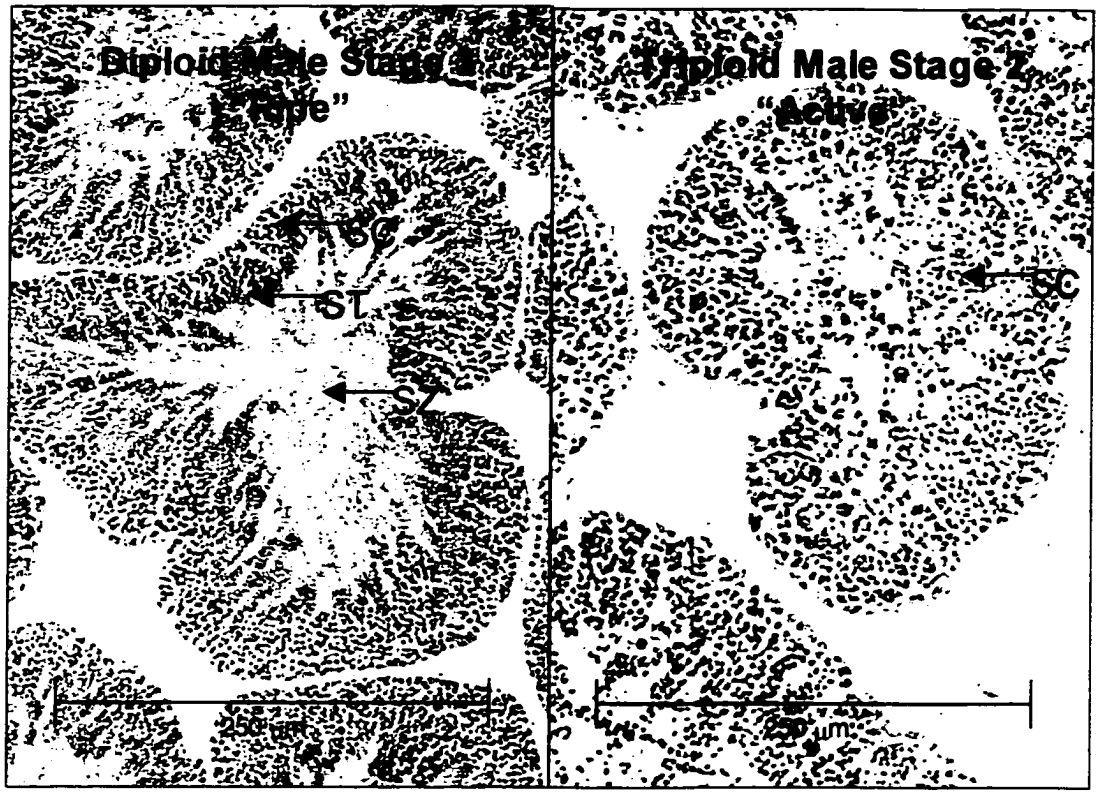

Figure 4.4: Photomicrographs of *Mytilus edulis* male gonad; 200X; H&E stain; diploid and triploid sampled from Lennox Island site May 1, 2000; SC = spermatocyte; ST = spermatotid; SZ = spermatozoa.

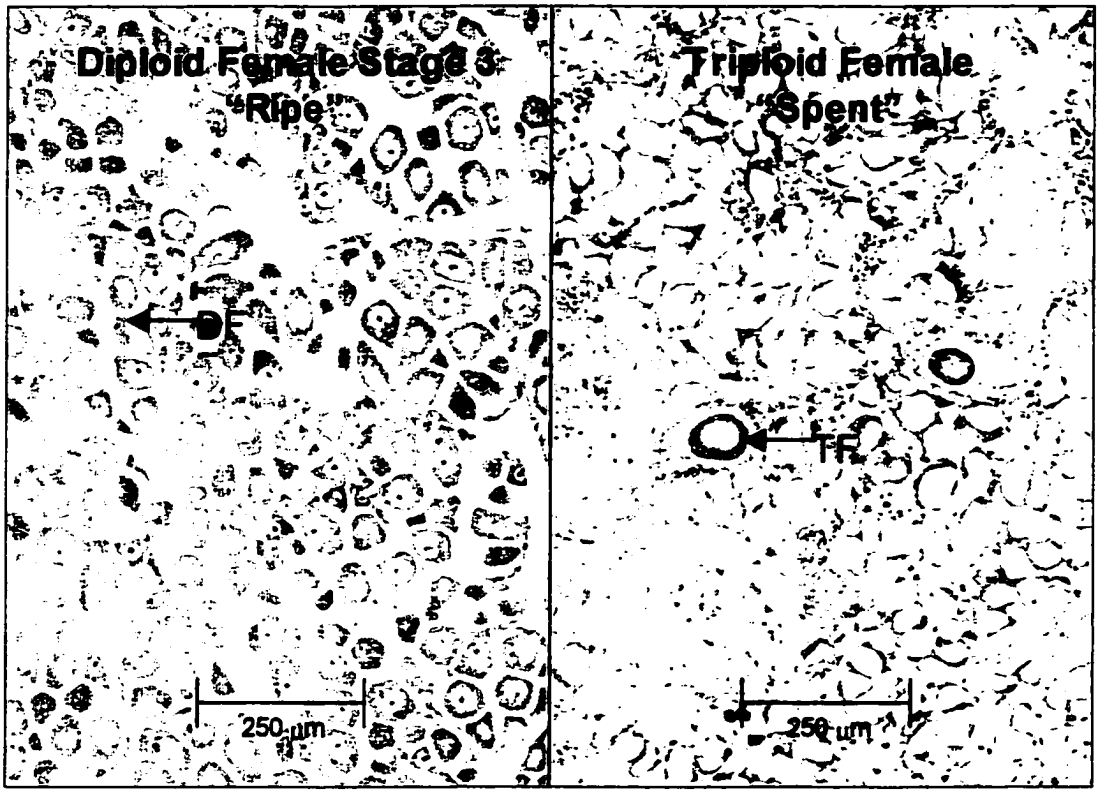

Figure 4.5: Photomicrographs of *Mytilus edulis* female gonad; 100X; H&E stain; diploid and triploid sampled from Lennox Island site May 1, 2000; DF = diploid follicle; TF = triploid follicle.

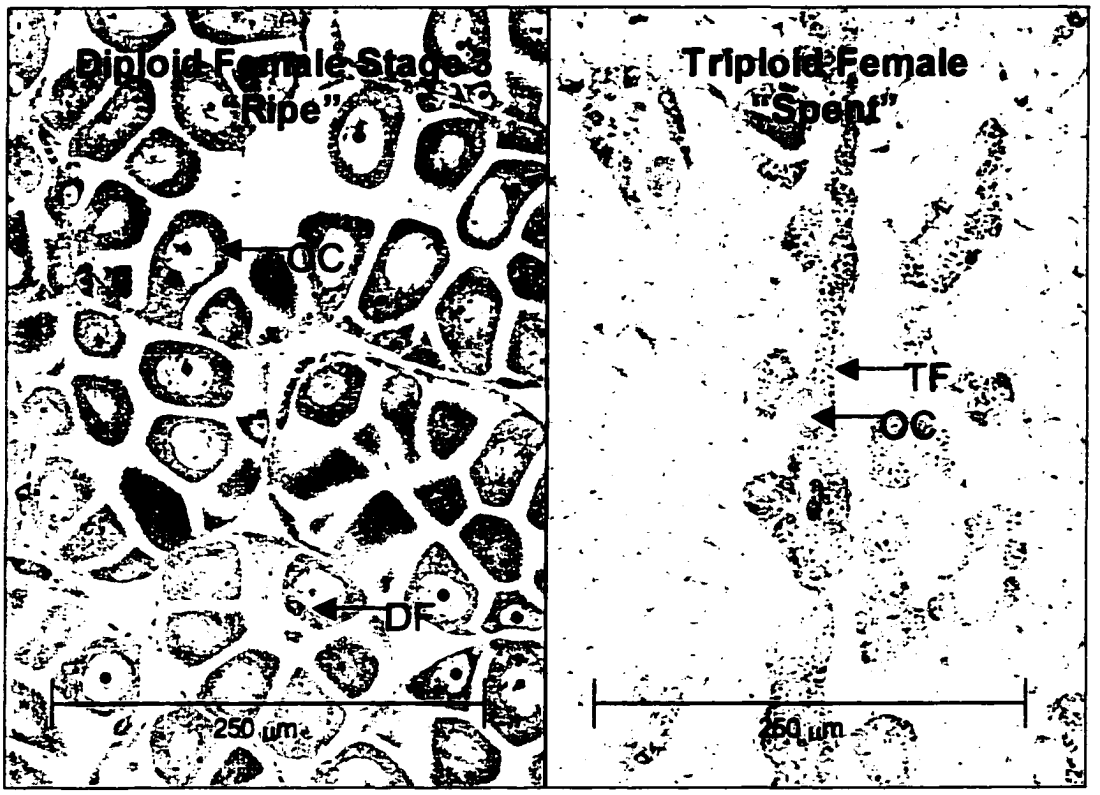

Figure 4.6: Photomicrographs of *Mytilus edulis* female gonad; 200X; H&E stain; diploid and triploid sampled from Lennox Island site May 1, 2000; DF = diploid follicle; TF = triploid follicle; OC = oocyte.

4.4 Discussion

4.4.1 Contamination of The Cage Units

Samples taken from the hatchery showed no triploid contamination of the diploids. Contamination likely occurred when deploying the animals, or while in the field. One possible explanation for this might have been the sieving of spat prior to deployment being ineffective in removing mussels smaller than the mesh size of the bags. If mussels smaller than 2 mm were in the bags when deployed they could have moved from one bag to another due to the close proximity of bags in the cage unit. This is the most likely explanation as some small mussels were found on the outside of the bags when cleaned and sampled after deployment.

Contaminated samples could have still been used for study. However, this along with the increasing difficulty in maintaining the cage units at all sites caused them to be eliminated from the study.

4.4.2 Growth

4.4.2.1 St. Peter's Bay

The percent triploid of experimental animals sampled from the experimental sock was low in both samples from the St. Peter's Bay site. Although experimental animals were greater than 80% triploid when deployed there were only 10% and 28% triploid when sampled in July of 2000 and June of 2001 respectively. There is no known explanation for this, although there might have been some environmental stress at some time that could have caused differential drop off from the experimental sock early in the field trial. The CI was lower for triploids (although not statistically significant) during the first sampling in July of 2000, indicating that triploids were at a slightly lower physical condition. Although not specifically investigated, there was no notable difference in condition or mortality between diploids and triploids in the hatchery. This suggests that the differential CI observation (between triploids and diploids) was the result of factors occurring after deployment at the St. Peter's Bay site. If a stressful event such as a sudden drop in salinity caused many of the animals in poor condition to drop off the sock, and if the triploids were in a lower physical condition at the time, then it is possible that more triploids were lost. This would have reduced the percent triploid on the experimental socks. The drop in percent triploid was therefore attributed to factors after deployment at the site.

Triploid mussels at the St. Peter's Bay site showed no significant growth advantages when compared to diploids during the first year of growth. In the second year however, triploids

had a significantly larger shell, had more total dry weight, and had more dry tissue mass compared to shell (higher CI). One possible explanation for the triploids outperforming the diploids in the second year of growth might be that sexual maturity was more pronounced at this site after the first year of growth. This was noted by observation of the gonads for relative size and texture during each sampling. One theory why triploids tend to grow faster than diploids is that unutilized reproductive energy, while the triploids are sterile, is placed into growth, while the diploids use energy for gamete production. If mussels at this site do not reach full sexual maturity during the first year due to slower growth, then by this theory there should be no growth advantage for triploids. It is known that mussels can mature in their first year of growth but the size at which this occurs depends upon the rate of growth (Bayne, 1976). Mussels at this site were sexually mature by the second sampling, and thus the growth advantage of triploids was observed. This suggests that neither triploid nor diploid mussels at the St. Peter's Bay site were producing any significant reproductive effort within the first year of growth. In the second year of growth however, diploids produced an appreciable amount of gametes that were shed during spawning prior to the second sampling. The triploids did not, and continued to grow, outperforming the diploids significantly in the second year. Previous results from the triploid pear oyster *Pinctada martensii* showed evidence of triploids outgrowing diploids solely due to a reallocation of reproductive effort (Jiang *et al.*, 1993). The authors used daily growth rates and compared growth during reproductive and nonreproductive months. Growth rates for both shell height and body weight in triploids were significantly higher than diploids during reproductive months, while there were no differences in the nonreproductive months. They concluded that the faster growth rate in triploids was caused by the retarded development of the gonad in triploids. Similar findings were reported in the catarina scallop by Ruiz-Verdugo (2000). They found that

the triploid growth advantage first became evident after approximately 3 months of growth, when diploid scallops were largely mature. These studies support the generally accepted idea that triploid shellfish show little difference from diploids before beginning sexual maturation (Guo and Allen, 1994). Beaumont *et al.* (1995) showed that triploid *Mytilus edulis* were significantly larger than diploids as adults, but not at the juvenile stage. The results from this site support those findings as smaller size (in year 1) triploids were not significantly larger than diploids, but became larger after the first year of growth. Triploid Mediterranean mussels, *Mytilus galloprovincialis*, were found to undergo some level of gametogenesis, although the maturation of gametes and level of gametogenesis appeared to be less than that of diploids (Davis, 1997). The current results for *Mytilus edulis* are in concordance with this study as the current study found gametogenesis at low levels in all triploids, but no mature gametes in any triploids.

4.4.2.2 Lennox Island

The percentage of triploid animals sampled from the experimental socks at the Lennox Island site was consistently as high or higher than upon the first deployment of the experimental animals. Therefore, there seemed to be no effect such as a possible differential drop off as might be suggested at the St. Peter's Bay site.

Triploid and diploid animals showed no significant difference in GSL after 3 months of growth at the Lennox Island site. After 9 months of growth however, triploids had larger shells, a larger total weight, and a larger CI, indicative of a higher general condition when compared to diploids. This sample was taken just prior to a spawn in the area, and sampled animals showed no evidence of being spawned out. After 11 months of growth at the site the difference between all growth measures except GSL increased, indicating that after the spawn in the area the

triploids were outperforming the diploids by a greater margin. This is likely due to the fact that many of the diploids would have spawned out a portion of their gametes while the triploids would have retained their whole body mass, increasing the difference between the two. This was confirmed in the histology as the 71% of diploids sampled for histology at this time showed at least some degree of spawning, while the triploids showed none.

The fact that triploids outperformed diploids within just nine months of growth at the Lennox Island site may indicate that mussels become sexually mature (and make growth differences between diploids and triploids evident) more quickly at this site. Mussels were sexually mature after just nine months of growth as evidence by the mature gonads seen in the histology sample taken at this time.

The remaining experimental and control socks at this site were accidentally harvested before they could be sampled again during the June 2001 sampling at St. Peter's Bay.

4.4.3 The Mechanism of Superior Growth in Triploids

There have been a number of postulated explanations for the superior growth of triploids. Polyploid gigantism has been suggested as one reason for triploids growing larger. In 1972, triploidy was proposed by as a genetic manipulation of potential value in fish aquaculture (Purdom, 1972). The theory was that both triploids and diploids would have the same number of total cells, but that the triploid cells might be larger, as their nuclei were larger due to the larger amount of DNA contained within. This was found to not be the case, as triploid fish cell numbers actually decline when cells become larger, making triploid and diploid fish the same size (Purdom, 1972). Cell nuclei of triploid shellfish are approximately 1.3 times the diameter of nuclei in diploids (Allen *et. al.*, 1989; Ekaratne and Davenport, 1993) but there are no published

reports indicating that overall cell size in somatic tissue is increased in triploid shellfish (Beaumont *et al.*, 1995). Polyploid gigantism might play a part in triploid growth increases however, and was hypothesized by Guo and Allen (1994) as being the reason that the dwarf surfclam, *Mulinia lateralis*, showed higher growth in triploids. The authors hypothesized that increased cell volume, which was observed, without a lack of cell-number compensation gave evidence that triploid growth superiority was caused, at least in part by polyploid gigantism.

Increased heterozygosity (causing "hybrid vigor") is another postulated reason for triploid shellfish superior growth. By this model a triploid simply has more genes, therefore having more hybrid vigor, and better growth. It has been suggested by Stanley *et al.* (1984) that triploid bivalves derived from meiosis I rather than by meiosis II treatments are more likely to show enhanced growth rate and viability since they inherit all genomic heterozygosity from the female (Shipigel and Spencer, 1996). Stanley *et al.* (1984) showed enhanced heterozygosity and growth rate of meiosis I American oysters, *Crassostrea virginica*, over meiosis II oysters and diploids during a three year growout period. Similar supporting results were reported by Hawkins *et al.* (1994) for the European flat oyster, *Ostrea edulis*. In terms of blocking meiosis I or II, triploids can be produced in either case, although commercially they are more commonly produced by blocking meiosis II as in general the percentage of triploids with lower mortality is obtained by blocking meiosis II rather than meiosis I (Beaumont and Fairbrother, 1991).

The last suggested mechanism for superior growth of triploids is that of a reallocation of reproductive energy into somatic growth. In this model energy usually diverted to gamete production is available for somatic growth in sterile triploid individuals. Thus, triploids grow faster. Davis (1988) calculated energy budgets for diploid and triploid Pacific oysters, *Crassostrea gigas*, and found that ripe yearling diploids were in a negative energy balance while

triploids of the same age remained in a state of positive energy balance. The result is that growth advantages become more pronounced in the reproductive season. In the Pacific oyster, *Crassostrea gigas*, Allen and Downing (1986) showed that triploid males produced one half as much, and triploid females produced one quarter as much gonads than did diploids. They found that triploids grew linearly through the period of reproduction, whereas diploids grew little until spawning when weight decreased by 64%. Subsequent to the diploids spawning growth of diploids and triploids was parallel as it was prior to the period of reproduction. There are many other studies which show that fast growth and high condition indices of triploid bivalves are probably due to partitioning of energy normally used for gamete production into somatic growth (Stanley *et al.*, 1984; Beaumont and Fairbrother, 1991; Jiang *et al.*, 1993; Ruiz-Verdugo, 2000). Whereas there have been differences as early as the larval stage shown to exist between diploid and triploid (Yamamoto *et al.*, 1988; Beaumont and Kelly, 1989), it is generally accepted that diploid and triploid shellfish are not appreciably different until beginning maturation (Guo and Allen, 1994). This model for the increased growth due to triploidy fits well with the current study as diploids at the Lennox Island site after 11 months of growth showed visibly more mature gonads during sampling than did those from the St. Peter's Bay site. Consequently, the triploids showed a growth advantage at the Lennox Island site (where maturity presumably caused triploids to grow better) whereas they did not at the St. Peter's Bay site. Also, at the St. Peter's Bay site diploids and triploids showed no significant differences in growth during the first year of growth. Triploids were significantly larger than diploids in all measures during the second year of growth when sampled during the reproductive season. The second year mussels would have been more mature and producing a larger proportion of gametes when compared to the first year mussels. Thus, reproductive energy reallocation into somatic growth made the difference

between triploids and diploids evident in the second year of growth. A similar finding was shown with the catarina scallop, *Argopecten ventricosus*, as a triploid growth advantage was first seen after 3 months of growth, when the diploid scallops were mostly mature (Ruiz-Verdugo, 2000).

It is possible that any or all three proposed mechanisms of triploid growth enhancement were acting to give the results of the current study. It is likely from the evidence in this case and previous studies however, that the reallocation of reproductive energy of diploids into somatic growth in triploids was a major factor in the superior growth of triploid *Mytilus edulis*.

4.4.4 Comparisons Between Sites and Other Investigated Bivalves

Triploid mussels at both test sites showed greater growth and higher dry tissue weights. At the St. Peter's Bay site there was no significant growth advantage after 11 months of growth, while the triploid mussels were significantly larger in every measurement after the same amount of time at the Lennox Island site. Previous experience from commercial mussel culture show a much higher growth rate at the Lennox Island site when compared to the St. Peter's Bay Site. More favourable growing conditions at the Lennox Island site may have provided the opportunity for the triploids to outperform the diploids, while less favourable conditions may have caused the triploids to perform poorly at the St. Peter's Bay site. This may be due in part to greater growth at the Lennox Island site causing a quicker reaching of sexual maturity. The larger and more mature a mussel is, the more energy goes into gamete production, and the more gametes it will produce as a proportion of its total biomass. This has been demonstrated in a number of studies (Thompson, 1984; Rodhouse *et al.*, 1986) showing that >90% of energy in large mussels can be channeled from somatic growth into reproduction. In these large animals reproductive tissues can account for up to 50% of the total soft body weight (Thompson, 1979;

Kautsky, 1982). If this is a larger number such as 50%, one would expect for the triploids to outperform the diploids even better. In general, the larger and more mature the animal, the more a triploid should outperform a diploid by virtue of the reallocation of reproductive energy theory. After 11 months of growth at the St. Peter's Bay site diploids had a 4.43% greater CI than did triploids. After the same time period at the Lennox Island site triploids had a 21.28% greater CI than diploids. The fact that triploids had a lower CI at the St. Peter's Bay site while triploids had a higher CI at the Lennox Island site (after 11 months) indicate that diploids outperformed triploids in relatively poor growing conditions during the first year of growth, suggesting that there is little growth advantage (and possibly a slight disadvantage) for triploids in a low growth area. By contrast, triploids outperformed diploids in relatively good growing conditions during the first year of growth. A similar finding for the Pacific oyster, *Crassostrea gigas*, was reported by Davis (1989) where triploids and diploids were grown in similar sites with two different temperature maxima. Triploids outgrew diploids in both sites, however the difference between diploid and triploids was greater in the site with a higher temperature maximum. Shipigel *et al.* (1992) also found that triploid *Crassostrea gigas* outgrew diploids and that the difference between the diploids and triploids were more pronounced at higher temperatures. This supports the findings in the current study that the level to which triploid growth varies from diploids appears to be related to the growout environment (ie. high versus low growth mussel leases). Although there is no temperature data available from the test sites of the current study, temperature, as in the aforementioned studies might have been a contributing factor to the variable triploid versus diploid performance.

Triploids have been produced in over 20 molluscs to date (Guo, 1999). In almost all species investigated, triploids demonstrate superior growth when compared to normal diploids.

Triploids grow 30-50% faster than diploids in most species, and 70-100% faster in others (Guo, 2001). Only one study on the softshell clam, *Mya arenaria*, has shown reduced growth in triploids compared to diploids (Mason *et al.*, 1988). Species showing higher growth in triploids include the eastern oyster (Stanley *et al.*, 1984; Barber and Mann, 1991), European flat oyster (Hawkins *et al.*, 1994), dalianwan oyster (Liang *et al.*, 1994), Pacific oyster (Guo *et al.*, 1996), Sydney oyster (Nell *et al.*, 1994), pearl oyster (Jiang *et al.*, 1991), bay scallop (Tabarini, 1984), catarina scallop (Riuz-Verdugo *et al.*, 1998) nobilis scallop (Lin *et al.*, 1995), dwarf surfclam (Guo and Allen, 1994), manila clam (Utting *et al.*, 1996), and hard clam (Eversole *et al.*, 1996). Davis (1997) showed there to be no advantages to triploidy in terms of growth or survivorship in Mediteranean mussels *Mytilus galloprovincialis*, in contrast to the present study.

The current study showed that triploid *Mytilus edulis* can outgrow diploids in a highly productive site in as little as 9 months and in a lower productive site in 22 months. The largest increase in growth when triploids are compared to diploids was after 22 months, during the reproductive period, in the St. Peter's Bay. Triploids had a 10.95% greater shell length, and a 62.82% greater dry tissue weight when compared to diploid controls. These numbers fit within the range of triploid growth advantages suggested by the aforementioned studies. Given the pattern of an increasing triploid growth advantage with growth at this site it would have been interesting to sample the more productive site in the second year of growth. One might hypothesize an even greater triploid growth advantage as it has traditionally higher growth and higher reproductive output (as evidence by traditionally larger drops in tissue weight after spawning, and traditionally faster growth) given the suggested theory that triploids grew larger due to a lack of reproductive energy utilization. Within the second year of growth in a relatively unproductive PEI mussel lease, and even with the first 9 months of a highly productive one,

triploids showed a significant growth advantage when compared to diploids, while showing no visible evidence of spawning. These findings suggest that triploid *Mytilus edulis* fit the required criteria of this investigation as not spawning during the spawning season, showing high soft tissue yields while showing superior growth when compared to diploids. As well, it is suggested by the differential site findings that triploid advantages might be factor or site specific. Any future work should address the causes and or relevant factors.

4.4.5 Histological Comparisons

Most mussels at the Lennox Island site were known to have spawned between the May 1 and July 24 sampling periods. This was observed by the site owner by a dramatic drop in meat weights when harvested weekly, and by buoys rising on the water, indicating a loss of weight on mussel lines. Prior to spawning all diploid mussels were observed to be in the ripe stage, with fully formed gametes. There were no fully formed sperm evident in the triploids. As well, the one putative female had scant oocytes and ovaries, both of which appeared retarded and abnormal. No triploids showed characteristics of being prepared to spawn in the near future.

In the July sample, 29% of all diploids were still in the ripe stage, showing fully formed gametes and had not spawned. The remaining 71% of all diploids showed some evidence of spawning. No triploids showed clear evidence of having undergone spawning to any degree, while all except for the putative female were in the inactive or active stage.

The sex ratio of the diploids show a clear pattern. In the May sample 4 of the 5 diploids were female (80% female) while 44 of the 45 triploids were easily distinguishable as males (98% male). These results were validated in the July sample when the diploids showed a sex ratio of 39% female and 61% male while the triploids again showed 99% were easily distinguishable as

males. This highly skewed male sex ratio in triploid mussels supports previous findings by Kiyomoto *et al.* (1996) in *Mytilus galloprovincialis*. The authors looked at the sex ratio and gametogenesis in triploid Mediterranean mussels. They found all triploid mussels to be male. They did identify 4 mussels containing a few oocytes in a group, and they termed these as being of unidentified sex. Those mussels were noted as having the gonads mostly reabsorbed and also as having all germ cells disappeared. This description best fits with respect to the putative females identified in both triploid samples in the current study. The authors also found no ripe animals in the triploid group, and suggested that their findings suggested that most spermatocytes in triploid gonads were at the prophase I of meiosis and that gametogenesis of triploid mussels was arrested at this stage. Although no attempts to stage meiosis in the triploid germ cells of the current study were made, the current findings are in concordance with those of Kiyomoto *et al.* (1996). The fact that no spermatozoa (a product of meiosis) were found in triploid gonads, and that only spermatocytes (a pre-meiosis germ cell) were present suggest that most gametogenesis was arrested in triploids prior to meiosis, possibly at prophase 1, as was suggested by Kiyomoto *et al.* (1996). The all male (save the putative females) triploid progeny also supports the previous author's suggestion of a possible genetic model for sexual determination in mussels similar to that of avian species.

Gametogenesis is suppressed, but differential among and within most triploid bivalve species. Triploid noble scallop *Chlamys nobilis* were completely sterile and produced neither mature spermatozoa nor oocytes (Komaru and Wada, 1988). Similarly, catarina scallop, *Argopecten ventricosus*, showed severely reduced fecundity, making fertilization not possible (although theoretically still possible under laboratory conditions) (Ruiz-Verdugo *et al.*, 2001). Shipgel et al. (1992) found that in the Pacific oyster, *Crassostrea gigas*, while 92% of observed

diploids ripened at elevated temperatures, 0% of triploids ever became ripe. Virtually all triploids in the soft shell clam *Mya arenaria* exhibited underdeveloped gonads with no mature gametes (Allen *et al.*, 1986). Reduced, but normal gametogenesis occurs in triploid Japanese pearl oyster *Pinctada fucata martensii* (Komaru and Wada, 1990), triploid dwarf surfclam *Mulinia lateralis* (Guo and Allen, 1994), and triploid Pacific oyster *Crassostrea gigas* (Akashige, 1990; Allen and Downing, 1990; Guo and Allen, 1994). Female gametes from triploid Japanese pearl oysters as well as both female and male gametes from triploid Pacific oysters were found to be capable of fertilization and development (Komaru and Wada, 1994; Guo and Allen, 1994). In the present study there were no normal fully formed gametes readily visible in any of the observed triploid mussels. This suggests that, as suggested by Kiyomoto *et al.* (1996) for *Mytilus galloprovinciallis*, triploid *Mytilus edulis* appear to not become appreciably ripe or spawn. Evidence for this is the absence of any observed spermatozoa in the triploid samples prior to and after the spawning event of May-June 2000.

There is also a notable alteration of the sex ratio in triploid bivalves. All triploid soft shell clams were found to be female (Allen *et al.*, 1986); in contrast, all triploid Mediterranean mussels were found to be males (Kiyomoto et al., 1996). A recent study has also shown the suppression of functional hermaphroditism in the catarina scallop, *Argopecten ventricosus*, causing a gradual replacement of the male gonads. The result was that 96% of all triploids could only be described as female, whereas all diploids remained hermaphrodites (Ruiz-Verdugo, 2000). By contrast, dwarf surfclam and Pacific oyster show no significant difference between sex ratio in diploids and triploids (Guo and Allen, 1994; Allen and Downing, 1990). These findings have lead to the proposal of several different genetic mechanisms for sex determination amongst the different investigated species. The results from the current study are in concordance with

those given for the Mediterranean mussel *Mytilus galloprovincialis*, suggesting that *Mytilus edulis* triploids perform similarly in terms of gametogenesis, and therefore may share the same sex determining mechanism.

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5. SUMMARY OF THE RESEARCH

5.1 The Suitability of Triploids as a Solution to a PEI Mussel Industry Problem

The PEI mussel aquaculture industry is interested in producing triploid mussels. In order for the technology to be useful it must fit certain criteria. The triploids must be relatively easy to produce, such that hatchery workers need not require specialized training. As well, the production must be efficient, reliably producing a high yield of triploids. The production of a high percentage of triploids is imperative to make the triploids useful to satisfy the spawning season market. Triploids cannot currently be separated from diploids by any commercially useful means. If there are many diploids within a triploid cohort there could be appreciable spawning within the cohort and effectively reduce the advantage for which they were created. A high survivorship is also important so that hatchery resources are used efficiently in production.

As well, the triploids must show growth similar to, or superior to standard diploids. Slower growing mussels would reduce the advantage of triploids by making the crop stay on the lease longer, requiring more care, and effectively lowering profit margins.

Lastly, triploids must not show any detectable spawning during the spawning season and they must retain their soft tissue mass during and immediately after the local spawning period. This would allow the marketing of the triploids during the period when product quality diminishes due to diploids spawning. If triploids are capable of spawning this would mean that there is little advantage for them during this niche market for which they were intended.

5.1.1 The Production of Triploid Mussels

The production of triploid *Mytilus edulis*, using the techniques investigated in this study, do not yield commercially acceptable triploid cohorts. Both the percent triploid (83.1%) and the

survivorship (1.39%) are too low. Further optimization will be required in order to make this technology useful for the commercial production of triploid mussels.

5.1.2 The Lack of Spawning Evidence in Triploids

The current study showed that while diploid siblings showed evidence of spawning by a low soft tissue weight, and by histological signs of a loss of ripe gametes, triploids did not. This was the paramount concern for the PEI mussel industry. It appears that triploid mussels do not spawn and retain a high level of soft tissue weight immediately after the spawning season. Thus they are at a high quality level during the time for which they were planned to be marketed. Although these results are encouraging, further work should be done to investigate this in a more detailed manner.

5.1.3 Growth Advantages of Triploids

Triploid mussels grew faster and larger than diploids. Triploid shell growth was higher. As well, triploids had a higher amount of soft tissue relative to the animal whole weight (condition index). This is an important measure of quality to the industry. Growing mussels faster and larger can help the grower realize benefits through a faster crop turnaround and less labour per unit of product. Thus the superior growth of triploids could also be of a benefit to growers. Triploid mussels meet industry requirements in this regard as well.

5.2 Relevance of the Research in the Context of the Current Knowledge of Triploids

The work in the current study contributes to the body of scientific knowledge on triploid bivalve molluscs. Some of these findings confirm previous studies, and others introduce new

scientific data on triploid *Mytilus edulis*, such as growth relative to diploid siblings and the in depth characterization of meiotic events prior to first cleavage of the embryo.

5.2.1 Description of Meiotic Events

The description of the meiotic events researched in this study is very useful scientifically. There are many references to the meiotic events in *Mytilus edulis*, but often with little detail. Most references state only that both polar bodies are extruded within the first hour of development, and that cleavage is also within the first hour. The minute by minute description (given by % eggs in a meiotic stage) shown in Figure 2.1, section 2.3.2, will be useful to research concerned with the specific times at which polar body 1 or 2 are extruded. Any future optimization of the induction technique can reference this for a better understanding of what stages they might be affecting with various treatments.

5.2.2 Induction Method

Triploid bivalve molluscs have been produced for 20 years, and in a number of species. Therefore, the production of triploid *Mytilus edulis* is not totally unexploited research. However, there has been little work on the induction of triploidy in *Mytilus edulis*. Several studies have produced triploid *Mytilus edulis*, but never with the ultimate goal of commercialization of the technique. The optimal technique provided from this research is not at a commercially relevant level, but is better than some listed in the literature. As well, only one study has attempted to use 6-DMAP as a triploidy inducer for *Mytilus edulis*. Thus, the current study gives an important starting point for future optimization of the technique using 6-DMAP.

5.2.3 Growth Differences

Again, the superior growth of triploid bivalve molluscs has been well documented for nearly two decades. Therefore, the fact that triploid mussels outgrew diploid siblings (section 4.4.2) in the same environment is not unexpected. This finding is important however, as it adds support to the scientific reports that triploid bivalve molluscs tend to outperform diploids in terms of growth.

One interesting finding was that there might be a site specific difference in the growth advantage of triploids. That is, triploids outperformed diploids at a faster rate in an area of high mussel growth when compared to an area of lower mussel growth. There is little published work in this area, and no known study specifically addresses it in terms of a high growth area versus a low growth area. Identifying the types of sites where triploids grow best is especially important for commercialization, which will require optimal performance to maximize benefits.

5.2.4 Relative Sterility and Sex Ratio

The current study investigated the spawning differences in triploid and diploid mussels by microscopic examination of the gonad. Triploids showed no evidence of spawning, while diploids did. Further evidence of the relative sterility of triploids was given by the large increase in the dry soft tissue weight of triploids compared to diploids after the spawning event (up to 62.82% larger in the triploids, see Table IV.III, section 4.3.1.1). Presumably, if the diploids spawned they lost some soft tissue mass while the triploids did not. This, at least in part, likely accounts for some of the dry tissue weight difference after the spawning event. The relative sterility and resulting benefits have been well documented for a number of triploid molluscs.

Thus, these findings are not novel, but strengthen the current scientific data on the relative sterility of triploidy in all bivalve molluscs.

The highly male-skewed sex ratio is an important finding in this study. Previous reports show various species of molluscs showing different sex ratio patterns due to triploid (section 4.4.5). This study supports the current data on triploid Mediterranean mussels, *Mytilus galloprovincialis*, which show a similar sex ratio. This observation, along with the other studies in this area will help to elucidate the sex determining mechanism(s) in molluscs. To date, several hypotheses have been published as a result of triploid work in a number of species. Data such as those presented in this thesis will help researchers to eliminate some hypotheses, and develop new ones.

5.3 Challenges Faced Throughout the Project – Suggestions

Throughout this project, as with any other, there were a number of challenges faced which should be mentioned. Any future work in this area could benefit from these experiences. These can be described as hatchery challenges, loss of larval samples prior to ploidy determination, and the loss of samples in the field.

5.3.1 Hatchery Challenges

The production of triploids in this study faced several challenges in the hatchery. The largest challenge was the water filtration system at the ESH. The large amounts of visible detritus in the filtered water caused into question the effectiveness of the filtration system. The introduction of pathogens or detritus to act as a surface area for bacterial growth may have been a factor in the subsequent loss of samples sent away for ploidy determination (section 3.4).

The production of an adequate amount of microalgae to feed the larvae and juveniles in this study was also problematic at times. The hatchery was essentially closed at the time. Therefore, all hatchery operations (including the time consuming production of large amounts of algae) were performed by one person. The use of a dried feed product was attempted to alleviate some of the need for large amounts of algae. Any future work involving the production of animals should ensure that a sufficient amount of microalgae can be produced while still allowing enough time for the proper care of the animals.

The location of the hatchery also caused problems in transportation. This was specifically important when some of the induction trials were being performed. Several times winter storms , and the coordinating of transportation for others to help in large experiments, led to scheduling problems. This will be a consideration for any future large-scale induction trials.

5.3.2 Loss of Larval Samples

The live shipping of samples for ploidy determination caused problems in some cases (section 3.4). A possible compromise in the filtration system might have compounded a loss of larvae during shipping by introducing harmful bacteria into larval cultures. Any future work with triploid larvae should maintain a focus on good husbandry practices. As well, sending samples across the continent for analysis might have also exacerbated the problem. It is strongly advised that any future ploidy analysis be done as closely as possible to avoid shipping time, and to allow for a quick processing of samples.

5.3.3 Loss of Field Samples

Several complications occurred in the field work of this study. One previously discussed (section 4.3.1) is that of the contamination of controls in the deployed cage units. As well, there were difficulties scheduling sampling with participating growers in some cases. Site accessibility should therefore be a major focus of site selection for any ensuing growth trials. The loss of the experimental and control socks at the Lennox Island site was also an unfortunate accident. These would have given more information on possible triploid performance differences based on site if they were sampled in June of 2001, during the St. Peter's Bay sampling period. However, site accessibility and grower cooperation were generally good in this study, and should be considered as major factors in any ensuing field trials.

5.4 Future Directions in This Research Area

In the current study triploid mussels have been shown to possess the characteristics desired to fill the spawning period marketing niche. Future work in this area should continue.

The main area of concern is optimizing the triploid induction technique to a commercial level. Commercial production of Mediterranean mussels, *Mytilus galloprovincialis*, produces 10-15% survivorship and 100% triploids reliably. This success level is the result of the optimization of the technique over time. The current optimal treatment for *Mytilus edulis*, with a survivorship of 1.39% and 83.1% triploid, is not at a comparable commercial level. Further optimizations should use the meiotic events description from this study to focus on targeting exact windows for treatment. As well, various levels of 6-DMAP should be tested in conjunction with heat shock. The goal should be to induce triploidy in a manner that subjects the embryos to as little toxic chemical, or lethally high temperature as possible to maximize survival.

A cost-benefit study would also be useful in order to determine what the true benefits, after an investment in a hatchery setup, would be to the industry. This would be the next logical step in order to determine the level of interest of the industry in realizing the commercial benefits of triploid mussels. More work will be required on the induction method and hatchery and rearing techniques before this can be assessed.

The indication in the current study that the growth benefits of triploids might be site related is also important to investigate. It may be that triploids perform exceptionally well in areas of high mussel growth, while performance is not as good in areas of marginal mussel growth. As triploid mussel production will likely be costly, the opportunity to maximize any benefits by increasing performance should be used in all cases. If triploid mussels perform better in a particular site type, then using those sites for their growout could yield significant growth advantages as well as the suitability for marketing during the natural spawning period.

Lastly, if the industry becomes committed to the idea of producing triploid mussels for a yearly niche market, then the investigation of tetraploids would be useful. Tetraploids, mated with diploids, produce 100% triploids without the use of chemicals. This would dramatically increase survivorship as well, as the embryos are not subjected to the 6-DMAP, which causes the low survivorship. Tetraploids can be crossed with other tetraploids in order to make more tetraploids, so a renewable broodstock could be developed. This is a method currently showing a large degree of success for the Pacific oyster, *Crassostrea gigas*.

5.5 The Potential Impact of This Project on The Local Industry

This project has demonstrated that triploid mussels can be readily produced using the tested methodologies. Triploid mussels did not show evidence of spawning, and therefore would

be suitable for the industry to market during the natural spawning period. Growth advantages of triploids versus diploids were shown in the current study, making them even more attractive to the local industry.

In order for the industry to realize any potential benefits to the industry as a result of this research, a long-term commitment to the development of production on a commercial scale must be realized. If successful, triploid mussels could become a significant niche market product for the local industry, adding value to the industry, while strengthening the market.