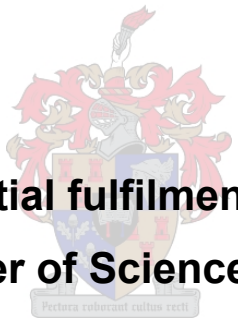
Induction of Triploidy in the South African abalone, *Haliotis midae*, by the use of hydrostatic pressure

by

Mathilde de Beer



Thesis presented in partial fulfilment of the requirements for the degree of Master of Science at the University of Stellenbosch.

Supervisor

Dr Danie Brink

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Abstract

The indigenous abalone, *Haliotis midae* has been a successfully cultured aquaculture species in South Africa since 1990. It has a slow growth rate and takes from two to five years to reach market size. Like for most other commercially important abalone species, the slow growth rate of *H. midae* is a cause of concern with regard to the profitability of farming and global competitiveness of the species.

Ploidy manipulation of the maternal genome, a universally growing practice in shellfish culture, is considered a promising method to improve the growth rate of abalone - a desirable trait in aquaculture organisms from a commercial perspective. This manipulation technique is employed to achieve sterility, which results in limited gonad development. The consequent re-allocation of resources to somatic growth results in improved growth.

The purpose of this study was to establish a viable method for the induction and validation of triploidy, on a commercial scale, in the South African abalone, *H. midae*. The focus was on hydrostatic pressure as a method of induction and flow cytometry as the method of validation.

The results obtained confirm hydrostatic pressure as an effective method for the induction of triploidy in *H. midae*, delivering high percentages of triploidy (>80%) over a wide range of pressures and times, in 48 hour-old larvae. Hydrostatic pressure had a negative effect on survival in 20 hour-old larvae. Flow cytometry was validated as a reliable, fast and accurate, though expensive, method for identification of triploidy in *H. midae*.

As an outcome of this study a manual of “Procedures for the Induction and Validation of Triploidy in the abalone” is presented (Appendix 1) together with recommendations for further studies on triploidy in the South African abalone, *H. midae*.

Opsomming

Die inheemse perlemoen, *Haliotis midae*, is sedert 1990 ‘n suksesvol gekweekte akwakultuur spesie in Suid-Afrika. ‘n Kenmerk van die spesie is die stadige groeitempo van tussen twee en vyf jaar ten einde bemarkbare grootte te bereik. Soos vir die meerderheid perlemoen van kommersiële belang, is hierdie stadige groeitempo rede tot kommer met betrekking tot die winsgewende kweek en wêreldwye mededingendheid van die spesie.

Die manipulasie van ploïdie van die moederlike genoom is ‘n toenemende praktyk in skulpvisboerdery en word gereken as ‘n belowende metode om die groeitempo van perlemoen te verbeter. Hierdie manipulasietegniek word gebruik om steriliteit te verkry wat manifesteer as onderdrukte ontwikkeling van die geslagsklier. Die gevolg is die herkanalisering van bronne na somatiese groei.

Die doel van hierdie studie was om ‘n lewensvatbare metode vir die induksie van triploïdie op ‘n kommersiële skaal in die Suid-Afrikaanse perlemoen, *H. midae,* te vestig. Daar is op hidrostatiese druk as metode vir die induksie en vloei-sitometrie as metode vir die geldigverklaring van triploïdie gefokus.

Die resultate van hierdie studie bevestig dat hidrostatiese druk ‘n effektiewe metode vir die induksie van triploïdie in *H. midae* is. Hoë persentasies van triploïdie (>80%) is oor ‘n wye reeks van drukke en tye in 48 uur oue larwes verkry. Daar is gevind dat hidrostatiese drukbehandeling ‘n negatiewe effek op die oorlewing van 20 uur oue larwes het. Vloei-sitometrie is bevestig as ‘n betroubare, vinnig en akkurate, maar duur metode vir die identifikasie van triploïdie in *H. midae*.

As ‘n uitvloeisel van die studie word ‘n handleiding “Procedures for the Induction and Validation of Triploidy in the abalone” (Appendix 1) aangebied tesame met aanbevelings vir verdere studies rakende triploïdie in die Suid-Afrikaanse perlemoen, *H. midae.*

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CHAPTER 1: Literature Review

1.1 General background

Since the earliest civilizations, communities all over the world have practiced agriculture, "the science or practice of cultivating the land and keeping or breeding animals for food" (Oxford Advanced Learners Dictionary of Current English, 1998, S.v. 'agriculture'). With the passing millennia, agriculture has become increasingly important to meet the demands of a growing population. As defined by the United Nations Food and Agriculture Organization (FAO), aquaculture is the "farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants" (White, O'Neill and Tzankova, 2004). This form of agriculture has been practiced since the earliest record of human history, dating back over 4 000 years, but it is only over the last 50 years that aquaculture has developed into a worldwide industry (Brink, 2001).

According to Van Oordt (1993) the rapidly expanding human population on the earth consumes increasing amounts of food, not only food derived from agriculture, but also food from natural aquatic ecosystems and from farms culturing algae, finfish and shellfish. Indeed, all over the world, but especially in Asian countries, including Japan and China, fish, molluscs and crustaceans form an important part of a protein-rich diet.

Figure 1.1 is a representation of the status of fish production by developing countries over the past two decades in relation to other selected agriculture commodities.

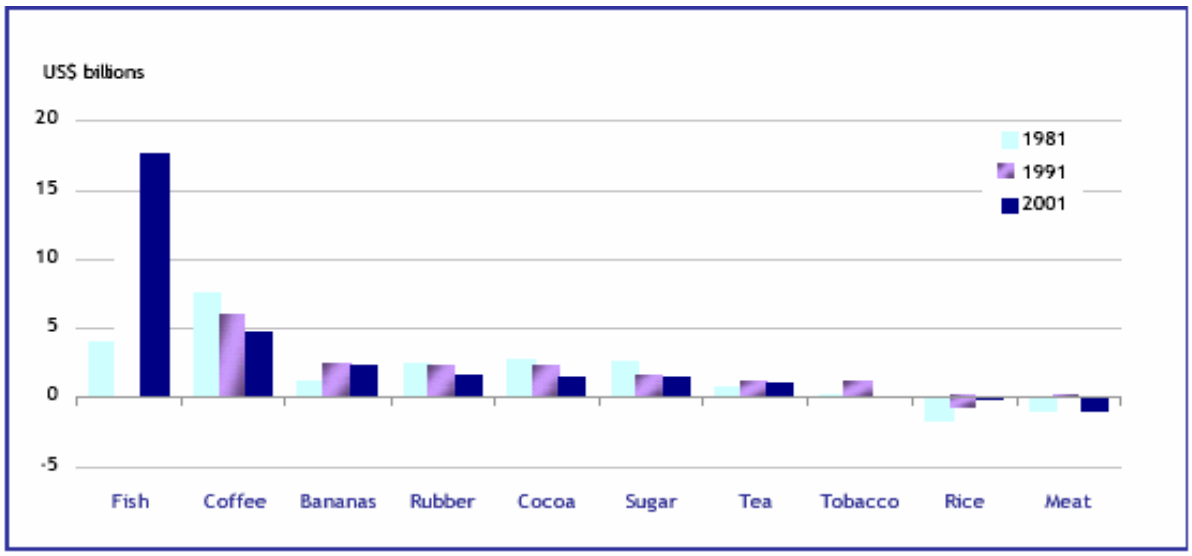

Figure 1.1 Net-exports of fish and selected agricultural commodities by developing countries (Vannuccini, 2003)

Van Oordt (1993) predicted an increase in the need for aquaculture products from 13 to 30 million tonnes per year by the year 2000. According to the Food and Agriculture Organization (FAO) the reported total aquaculture production for 2000 (including aquatic plants which accounted for 10.1 million tonnes) was 45.7 million tonnes by weight and US$56.5 billion by value. The FAO (2002a, b) reported that aquaculture's contribution to global supplies of fish, crustaceans and molluscs increased from 3.9 percent of total production by weight in 1970 to 27.3 percent in 2000, making aquaculture the fastest growing sector in animal food production. More than 210 different farmed aquatic animal and plant species were reported in 2000 (FAO, 2002a). Projections of world fishery production in 2010 range between 107 and 144 million tonnes and most of the increase in production is expected to come from aquaculture (FAO, 2002b). It is worth noting that molluscs represent a fast growing sector within aquaculture (Figure 1.2).

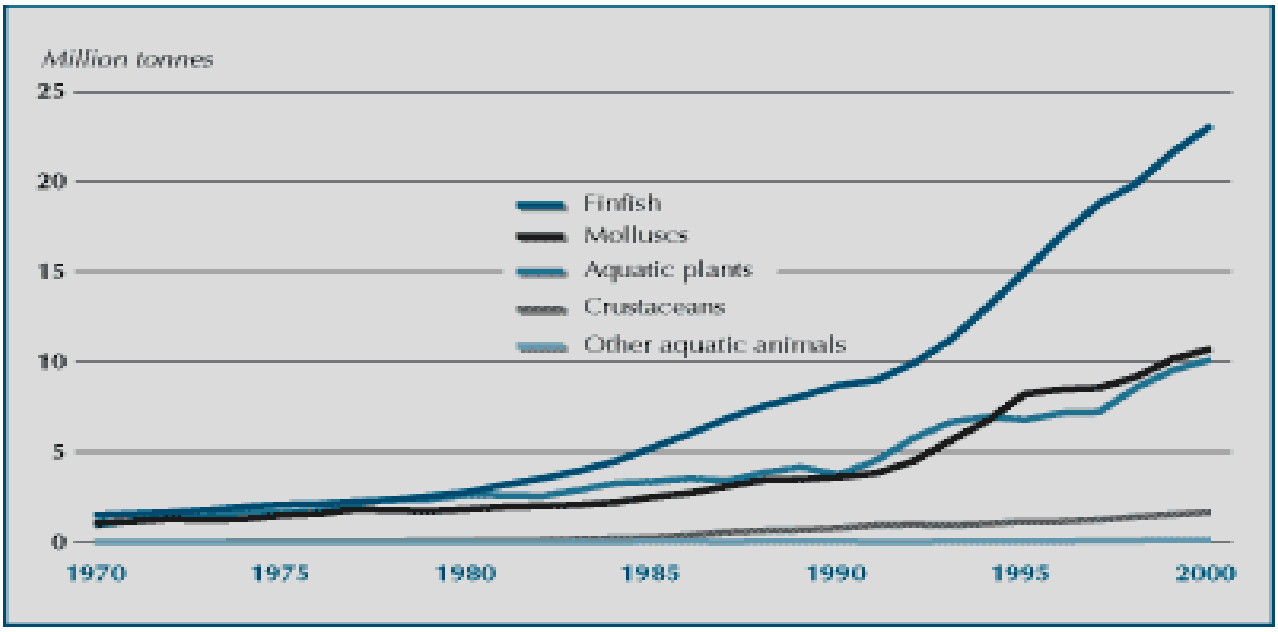

Figure 1.2 Trend of world aquaculture production by major species groups showing molluscs amongst the top three species groups with a steady increase in production over the past 30 years (FAO, 2002a)

Aquaculture in South Africa has also shown a significant increase over the past decade, with total production increasing by 31 percent in weight and 35 percent in value from 1998 to 2000 (Brink, 2001). According to production statistics for South African aquaculture for the year 2000, species with a high production value included abalone (production value R36 million), trout (production value R35.4 million), mussels (production value R5.14 million) and oysters (production value R5.1 million) (Brink, 2001).

Several advances have been made in the field of aquaculture technology in an attempt to meet the growing demand for aquaculture products worldwide. The development of a biotechnological basis for global aquaculture became imperative to support the quantitative increase in the culture of aquatic animals as well as to prevent ecological disorder in natural aquatic ecosystems (Van Oordt, 1993). Biotechnology has, in fact, played a revolutionary role in the genetic improvement of aquaculture species over the past two decades. Different approaches to genetic improvement include polyploidization, hybridization and selection (Boudry, Barré and Gérard, 1998). In most genetic improvement programs, the aim is to produce faster growing animals and so reduce the production time and cost for a market size individual (Elliott, 2000). Genetic manipulation, specifically ploidy manipulation of the maternal genome, has been used to improve growth rates in many shellfish species, including oysters, clams, mussels, scallops and some abalone species (Arai, Naito and Fujino, 1986; Cook and Stepto, 1998; Elliot, 2000; Purchon, 1977; Stepto, 1997). This technology shows great potential for application in commercial breeding (Boudry *et al.*, 1998).

1.2 South African abalone, *Haliotis midae*

Abalone form part of a group called Molluscs. The phylum Mollusca is only second to Arthropoda in number of named species in the animal kingdom and includes approximately 50 000 living species and 35 000 fossil species. It is a very diverse group, which includes species such as chitons, snails, abalone, oysters and octopuses (Hickman and Roberts, 1994).

Table 1.1 Taxonomic classification of the abalone (Hahn, 1989)

Phylum	Mollusca
Class	Gastropoda
Subclass	Prosobranchia
Order	Archaeogastropoda
Family	Haliotidae
Genus	Haliotis

Haliotids belong to the order Archaeogastropoda which is the oldest and most primitive group of prosobranch gastropods (Muller, 1986; Purchon, 1977). There are six haliotid species that occur in southern African waters, namely *H. midae*, *H. parvum*, *H. spadicea*, (Donovan), *H. queketti* (Smith), *H. speciosa* (Reeve), and *H. pustulata* (Reeve) (Hecht, 1994; Muller, 1986). *H. midae,* known locally as 'perlemoen', occurs along the Western and Eastern Cape shores of South Africa, and is the only abalone species of commercial importance in South Africa. The other five species of abalone are relatively small and not harvested commercially (Henry, 1995).

Strict conservation measures were implemented from 1965 to prevent over-fishing of *H. midae* (Genade, Hirst and Smit, 1988). In this year, the highest abalone harvest ever was reported at an annual catch of 2800 tonnes. In 1968 a maximum production quota of 386 tonnes was imposed and this was reduced to 227 tonnes in 1970 (Tarr, 1992). Due to continued concern over the state of the resource, the production quota was reduced to 181 tonnes in 1971. From 1979 to 1982 it was even further reduced by 10 percent to 163 tonnes. After this, the control system was changed to a whole mass quota and continuous efforts were made to manage this resource (Tarr, 1992). Years of

uncontrolled commercial fishing and poaching has brought the South African abalone, *H. midae,* to the brink of extinction (Lindow, 2003).

1.2.1 General Anatomy

All haliotids, including *H. midae*, are large, herbivorous, marine gastropods with a depressed shell, enlarged body whorl and reduced spire, near the back of the shell. The round or ear-shaped shell is characteristically perforated by a line of small respiratory pores located along the left margin of the shell. The older pores close successively as growth proceeds (Genade, Hirst and Smit, 1988; Hahn, 1989; Muller, 1986). The shell mouth is expanded to an extent that it almost covers the whole of the lower surface. This flat shell, which reduces resistance to waves, and wide shell-mouth, which enables the animal to attach firmly to the substratum, reflect adaptation of *Haliotis* to conditions of strong wave action (Global Ocean, 1995).

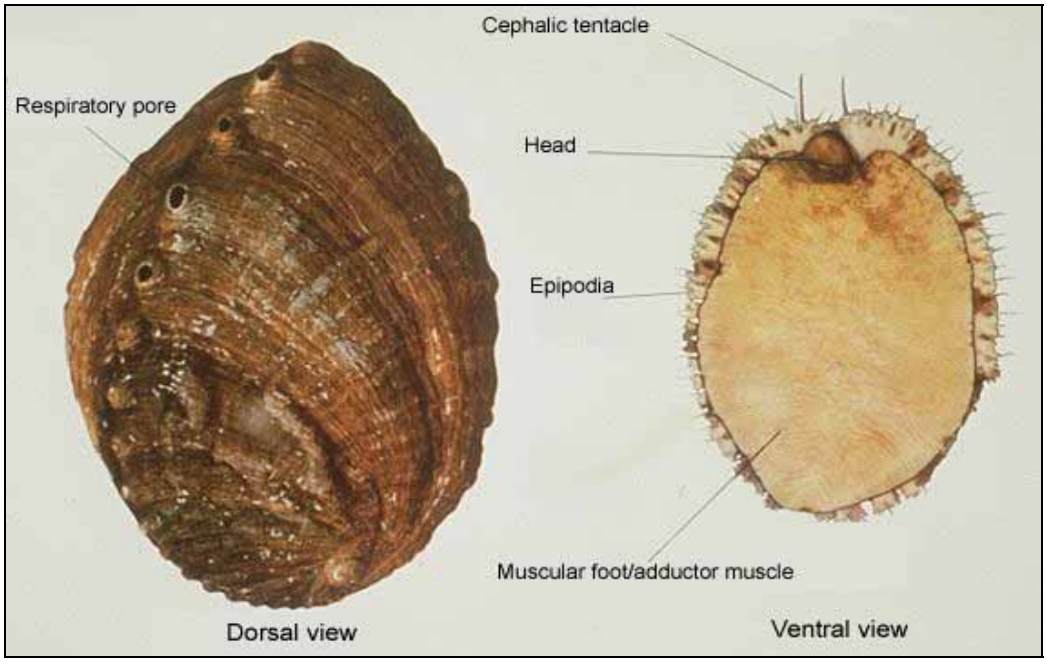

Figure 1.3 Dorsal and ventral views of the abalone (Sorgeloos and Co., 1997)

Underneath the shell lie the anterior head, a large muscular foot and the soft body which is attached to the shell by a column of shell muscles. The muscular foot is encircled by the mantle and the epipodium – a sensory structure bearing the tentacles. The epipodium, which projects beyond the shell edge, has a smooth or pebbly surface with a frilly or scalloped edge, and is a reliable structure for identifying abalone species

(FishTech, 2001). The foot is the edible part of the animal and can account for more than a third of the animal's weight. It is used by the animal to attach tightly, by suction, to rocky surfaces (Department of Fisheries, 2001). The organs arranged around the foot and under the shell comprise of a pair of eyes, a mouth with a long tongue called the radula, an enlarged pair of tentacles and the crescent-shaped gonad. Next to the mouth, and under the respiratory pores, is the gill chamber where water is drawn in under the edge of the shell and flows over the gills and out the pores, carrying waste and reproductive products out in exhalant water (FishTech, 2001). The abalone has a heart on its left side and blood, called hemolymph, flows through the arteries, sinuses and veins. The central nervous system lacks a concentration of ganglia into complex organs, although distinctive ganglia do occur in the head (Hahn, 1992). Because it has no obvious, organized brain structure, the abalone is considered a primitive animal (FishTech, 2001).

Abalone are dioecious animals and have a single gonad, either ovary or testis, enveloping the digestive gland, which forms the bulk of the visceral mass (Henry 1995; Newmann, 1967; Purchon, 1977). The gonad constitutes up to 15 - 20 percent of the soft body mass during the breeding season and remains this size until spawning, after which it rapidly decreases in size (Hahn, 1989; Henry, 1995). The gonad of female abalone varies in colour, ranging from brown (*H. iris*) (Wilson and Schiel, 1995), to green or blue-green (*H. asinina, H. australis* and *H. midae*) (Capinpin, Encena and Bayona, 1998; Fallu, 1991; Henry, 1995; Shepherd and Laws, 1974; Wilson and Schiel, 1995). The gonad of the male is white or cream coloured when ripe (Capinpin *et al.,* 1998; Fallu, 1991; Henry, 1995; Shepherd and Laws, 1974; Wilson and Schiel, 1995). Immature abalone have grey gonads (Fallu, 1991). The combined structure, which results as the gonad envelopes the gut, is called the conical appendage (Fallu, 1991; Hahn, 1989; Henry, 1995; Hooker and Creese, 1995). This structure is developed extensively to the right side of the body and around the right posterior margin of the adductor muscle (Henry, 1995). The gonad consists of a large lumen, bounded by germinal epithelium, with a connective tissue base which is well supplied with blood vessels (Newmann, 1967).

The ovary forms a series of chambers, separated by trabeculae that lie between the ovarian wall (outer epithelial layer of the conical appendage) and the wall of the digestive gland (Hahn, 1989; Henry, 1995; Newmann, 1967). The trabeculae are sheets of connective tissue which support the germinal epithelium, the site of egg production. The lumen of a fully developed ovary is filled with large eggs, imbedded in a gelatinous matrix,

before spawning. In the male, the lumen of the testis is traversed by tubes of connective tissue containing blood sinuses. The outer surfaces of these tubes are lined with germinal epithelium, which is the site of sperm production. When fully mature, the whole testis lumen is packed with sperm prior to spawning (Newmann, 1967).

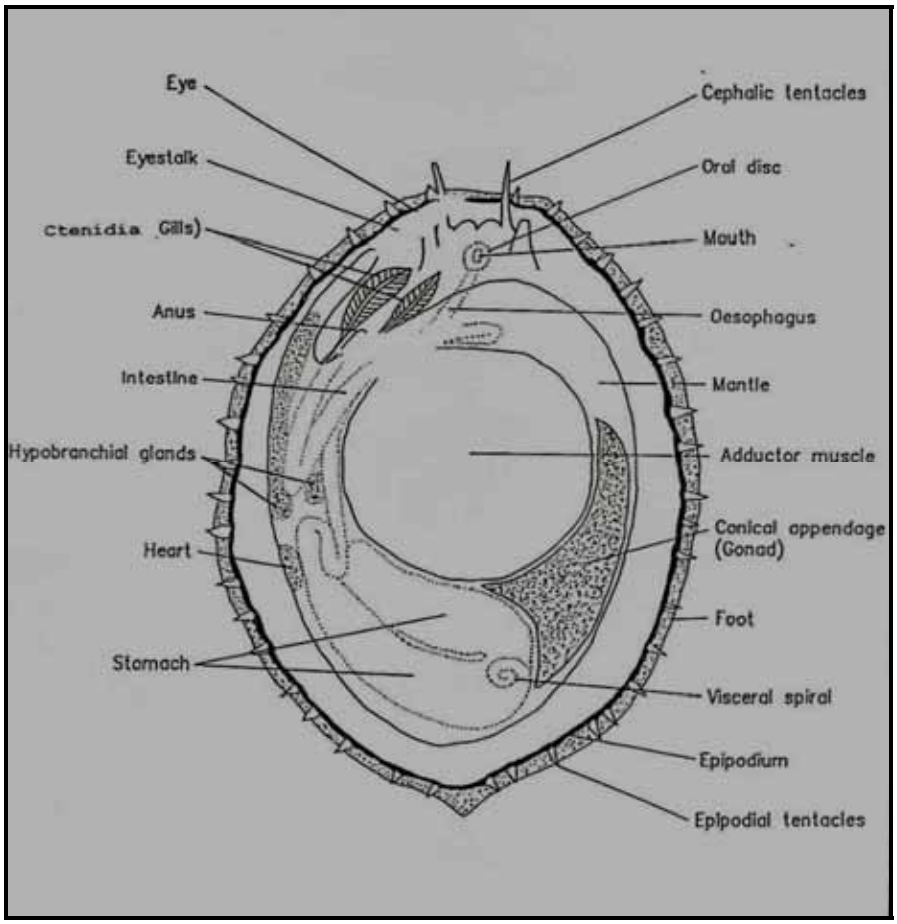

Figure 1.4 Anatomy of the abalone (Henry, 1995)

H. midae is the largest of the South African abalone species and has a reddish shell colour. The foot is pale cream to mottled light brown and tentacles and gills are yellow (Muller, 1986). The gonad of the female is green and that of the male, cream coloured when ripe. *H. midae* can reach a size of 90 mm shell length in six years and a maximum size of about 200 mm shell length at an age of over 30 years in the wild (Hahn, 1989; Sales and Britz, 2001).

1.2.2 Reproduction

Concerning reproduction, various modern lineages, including *Haliotis,* retained the most primitive condition. Being dioecious animals with a primitive reproductive system, the reproductive function of the adult abalone is limited to emission of large numbers of gametes into the environment and no protection or special provision is made for the developing embryos (Purchon, 1977).

An adult or sexually mature individual is defined by Hahn (1989) as “having either spermatozoa or primary oocytes”. Sexual maturity is reached within three to five years, depending on the species (Hahn, 1989). For *H. midae*, 100 percent sexual maturity may occur at around 7.2 years (80 - 105 mm shell length) in the wild and the shell length at sexual maturity is probably related to the temperature regime of the area (Barkai and Griffiths, 1988; Henry, 1995; Tarr, 1995). The mature abalone enters regular reproductive cycles in synchronization with the rest of the population. The reproductive cycle is defined by Hahn (1989) as the “time interval between successive spawnings in a population.” It is important for the reproductive cycles of individuals within a population to be relatively in phase, in order to have simultaneous or mass spawning. Because external fertilization is dependant on numbers of released gametes, higher fertilization success is accomplished with better synchronicity amongst individuals (Hahn, 1989).

The female reproductive cycle can be divided into four events: 1) Initiation of gametogenesis, 2) vitelogenesis, 3) oocyte growth and 4) spawning. Gametogenesis is initiated by the release of ripe eggs. Vitelogenesis and oocyte growth are both initiated by a change in water temperature or increased food supply. Gametogenesis, in both female and male abalone, entails the development of gametes until ripe when they are spawned (Hahn, 1989).

Spawning

Spawning is defined by Fallu (1991) as “the act of shedding sperm or eggs” (see Figures 3.2 and 3.3). Abalone exhibit the most primitive condition of spawning within the Mollusca. Ova and sperm are emitted in the exhalent water current and fertilization occurs at random in the surrounding seawater (Purchon, 1977). This is also called broadcast spawning or broadcast fertilization (Henry, 1995; Hooker and Creese, 1995).

Triggers for spawning

It is important for all abalone on a reef to spawn more or less simultaneously, as a maximum level of fertilization is dependant on high concentrations of eggs and sperm in the water (Fallu, 1991; Purchon, 1977). Abalone sperm are capable of fertilizing the egg for 4 - 5 hours, but due to turbulence in the water, it is essential for the sperm to find the egg within minutes (Fallu, 1991; Genade, Hirst and Smit, 1988; Hahn, 1989). Certain environmental triggers cause simultaneous spawning in a population. A sudden change in water temperature, exposure to air during low tide, photoperiod, lunar cycle, release of gametes from other individuals, surface winds of certain strengths, physical disturbances, food supply, genetic and hormonal factors and heavy surf, or a combination of these, can all be triggers for the induction of spawning (Fallu, 1991; Hahn, 1989; Purchon, 1977; Wilson and Schiel, 1995). According to Newman (1967) in *H. midae*, spawning is associated with a well-defined increase in water temperature.

The trigger mechanism, by which all abalone spawning events forms a cascade effect, is one where the first spawned gametes from the first spawning individuals stimulate the rest of the population to spawn. This mechanism relies on a hormone, called diantlin, which causes relaxation of the adductor muscle, enlargement of the openings of the respiratory pores and increased ciliary pumping of water by the respiratory pores. Thus, with a higher concentration of gametes in the water, still more animals will be stimulated to spawn and a higher percentage of fertilization will result (Purchon, 1977).

Sequence of events

The actual process of spawning begins when the abalone's posterior end touches the container/substrate and the anterior is raised. The shell is lifted (approximately five to ten minutes prior to spawning in the female abalone) to an extent where the gonad is visible (Hahn, 1989). The adductor muscle contracts rapidly and these contractions compress the conical appendage between the foot and the shell, resulting in the gametes being released from the respiratory pores (Hahn, 1989; Henry, 1995). Upon spawning, the gametes are passed via a single gonad duct directly into the kidney and from there, via the nephridiopore duct, out of the respiratory holes in the shell (Purchon, 1977).

In the female, germinal vesicle breakdown of mature oocytes in the ovary only starts after spawning has been induced. The oocytes are in the middle of the first meiotic reduction division when spawned from the gonad. Because a genital duct is absent in

abalone, gametes enter the right kidney through a longitudinal slit in the roof and exit through the renal duct into the gill chamber from where they are carried by the water currents out of the respiratory pores. Mature eggs are extruded loose from each other, while immature eggs come out in clumps (Hahn, 1989). Spawned eggs have a diameter of 200 - 250 µm and are negatively buoyant, causing them to sink to the bottom in quiet water (Fallu, 1991; Hahn, 1989; McShane, 1992; Newmann, 1967). Eggs significantly expand in volume over time after spawning, probably due to hydration of the egg content (Huchette, Soulard, Koh and Day, 2004). Spawned sperm are much smaller with a width of 1 – 1.5 µm and a length of 6 µm. The mature sperm consists of three parts: 1) the head, an elongated cone with an acrosome at the tip 2) the middle segment, cylindrical, 1 µm in width and 8 µm in length and 3) a filament/flagellum, about 50 µm long, that propels the sperm (Hahn, 1989; Henry, 1995; Newmann, 1967). Males are usually first to spawn. This stimulates the females to release their ova into the suspension of sperm (Henry, 1995). For this reason it is common for female abalone to orient themselves at higher places on the reef. The released ova can then sink through the cloud of sperm (Huchette *et al*., 2004).

Abalone, like most molluscs, are diploid – having two sets of chromosomes (Beaumont and Fairbrother, 1991). The diploid chromosome number (2n) of *H. diversicolor* is 31 - 32 (Yang, Chen and Ting, 1998) and that of *H. discus hannai* 36 (Arai *et al*., 1986). Sexual reproduction is a process where germ cells undergo two maturation divisions during the process of meiosis before becoming gametes (see Figure 1.5). Halving of the chromosome number occurs at the end of Meiosis I when one chromosome from each homologous pair goes to each daughter cell. Meiosis II is essentially the same as mitosis where complete replication and division of each chromosome into two daughter chromatids takes place. The result is that each daughter cell receives one chromatid. Male germ cells produce four gametes during meiosis, while female germ cells produce only one gamete. This is because female germ cells divide unequally. The products of the first maturation division are two cells with the same chromosomal constitution, but the one receives almost all of the cytoplasm. This cell is called the secondary oocyte. The other is called the first polar body. During Meiosis II the secondary oocyte undergoes the second maturation division and forms another polar body, called the second polar body, which again has the same chromosomal constitution as the oocyte, but very little cytoplasm. Neither of the polar bodies normally contributes

any chromosomes to the final zygote – which results from the fusion of sperm and ovum (Beaumont and Fairbrother, 1991).

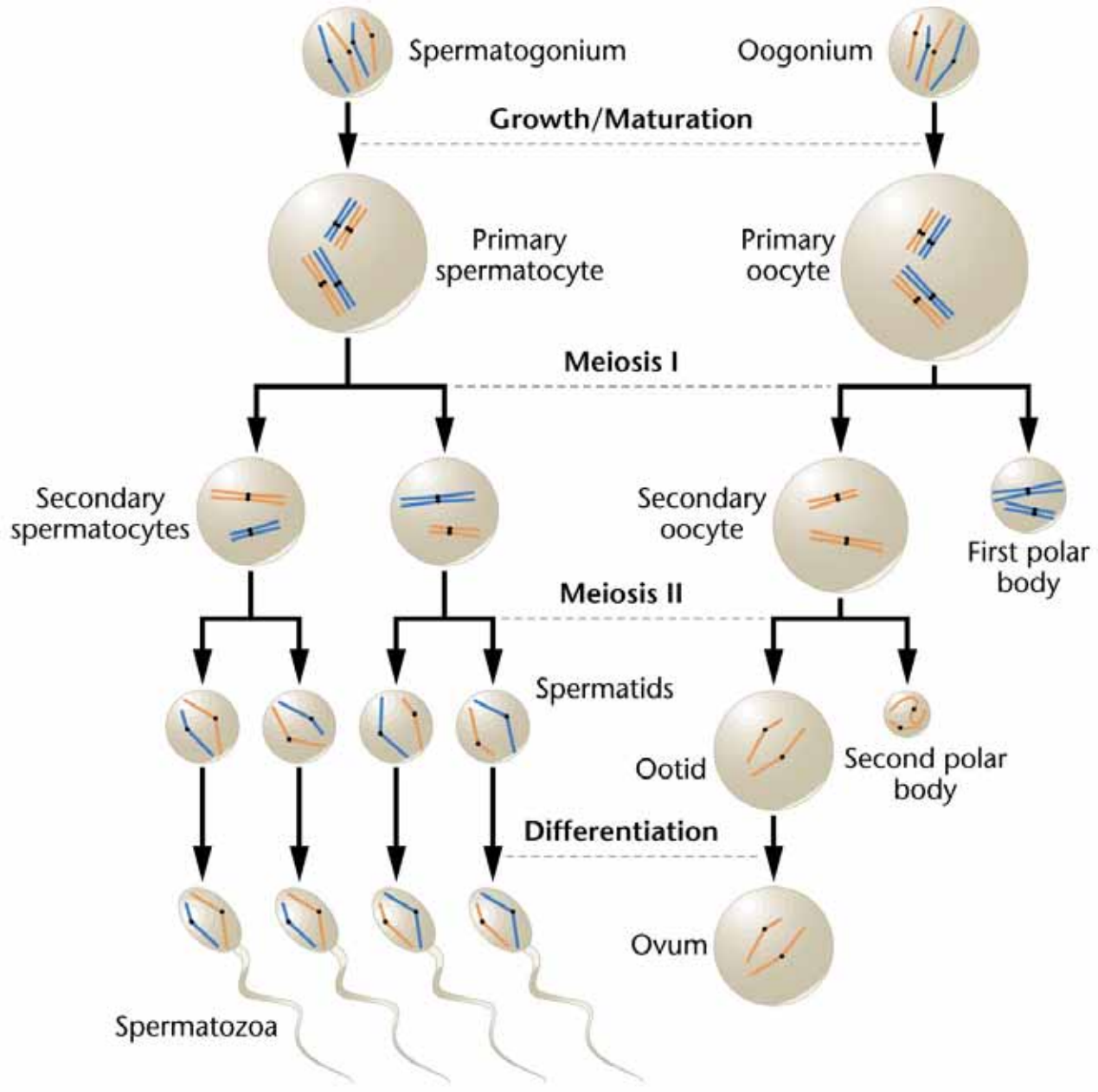

Figure 1.5 Gametogenesis, the process by which haploid gametes are produced, resulting in four spermatozoa in males and one ovum in females

Mature eggs are arrested at the prophase of Meiosis I and complete Meiosis I and II only after fertilization (Guo, Cooper, Hershberger and Chew, 1992). Polar bodies 1 and 2 are thus released after fertilization and polar body release is, in fact, described as stages in larval development (Guo, Cooper, Hershberger *et al*., 1992; Hahn, 1989).

Fertilization

Abalone reproduce by broadcast fertilization – after the eggs and sperm have been spawned, fertilization takes place at random in the surrounding seawater (Henry, 1995; Hooker and Creese, 1995; Purchon, 1977).

Additional to the synchronization of female and male spawning, other important factors determine the success of fertilization. One such factor is the fertilizability of the eggs. Two determinants of egg fertilizability are fecundity of the animal and the ratio between yolk diameter and total egg diameter. Egg biochemical content is size dependant, and therefore egg diameter is an important key in determining fertilizability. However, bigger egg size does not necessarily result in better fertilization performance. It was found in *H. rubra* that it is rather the yolk size: egg size ratio that determined performance and that good performance is restricted to a certain range of egg sizes (Litaay and Da Silva, 2001). Research by Huchette *et al*. (2004) confirmed that larger cytoplasm diameter offered more sites for the sperm to enter the cytoplasm and fertilise the egg successfully.

Sperm density also plays an essential role in the success of fertilization. Clavier (1992) found that no cleavage resulted when less than 1000 sperm per millilitre were used to fertilize eggs from *H. tuberculata*. Concentrations of 10^5 sperm per millilitre resulted in a hundred percent fertilization rate, but more than 10^5 sperm per millilitre resulted in lysis of the vitelline envelope and destruction of the ova. According to Huchette *et al.* (2004), fertilization success increases with sperm concentration and optimum sperm concentration, in a hatchery situation, was found to be approximately 10 000 - 200 000 sperm per egg.

Galindo, Moy, Swanson and Vacquier (2002) described sperm-egg interactions in the abalone as beginning when the sperm makes contact with the vitelline envelope and ending with fusion of the two gametes and incorporation of the sperm nucleus into the egg's cytoplasm. Upon encountering the abalone egg, the abalone sperm swims through the egg's first protective barrier, a thick gelatinous coat (Figure 1.6 A). The next, more formidable barrier is the 0.6 µm thick vitelline envelope (Kresge, Vacquier and Stout, 2001). The vitelline envelope consists of glycoprotein fibres, 64% carbohydrates and 36% protein (Galindo *et al*., 2002; Lewis, Eickhoff and Stringham, 1992). These vitelline envelope fibres are tightly intertwined and held together by hydrogen bonds (Galindo *et al*., 2002). The vitelline envelope represents a physical block to fertilization that the sperm

must overcome to accomplish fusion with the underlying plasma membrane (Lewis *et al*., 1992).

Contact between the sperm and the vitelline envelope induces the exocytotic acrosome reaction (Kresge *et al*., 2001) (Figure 1.6 B). The sperm's acrosome releases lysin, a 16-kDa nonenzymatic cationic protein onto the surface of the vitelline envelope. Lysin dimers then bind to a giant glycoprotein (1000 kDa) named vitelline envelope receptor for lysin (VERL). Dimer-dimer binding results in tight species-specific binding of lysin to VERL (Galindo *et al*., 2002). The NH_2 terminal of lysin is mostly involved in the specificity of this binding, while the $COOH^-$ terminal may be involved in the dissolution of the vitelline envelope (Lewis *et al*., 1992).

Upon binding, lysin causes the vitelline envelope fibres to unravel and splay apart, creating a hole in the vitelline envelope where the sperm can go through (Galindo *et al*., 2002; Kresge *et al*., 2001) (Figure 1.6 C).

Simultaneous with the exocytotic acrosome reaction is the formation of a 7 μm long acrosomal process coated with acrosomal protein at the tip of the sperm. Upon passage through the vitelline envelope, the tip of this process fuses with the egg's plasma membrane. This fusion of male and female gametes activates the egg's contractile cytoskeleton to draw the sperm into the egg cytoplasm (Figure 1.6 D). Fusion of the male and female pronuclei results and the egg is activated to begin cleavage (Kresge *et al*., 2001).

Abalone oocytes are highly resistant to polyspermy, because of a rapid electrical polyspermy block. At 1.75 minutes after sperm is introduced to oocytes, the membrane potential rises from -45 mV to +35 mV in less than 1 second. This block is postulated to take place at the egg plasma membrane and not the vitelline envelope, as sperm can still be found in the perivitelline space after fertilization is completed (Stephano, 1992).

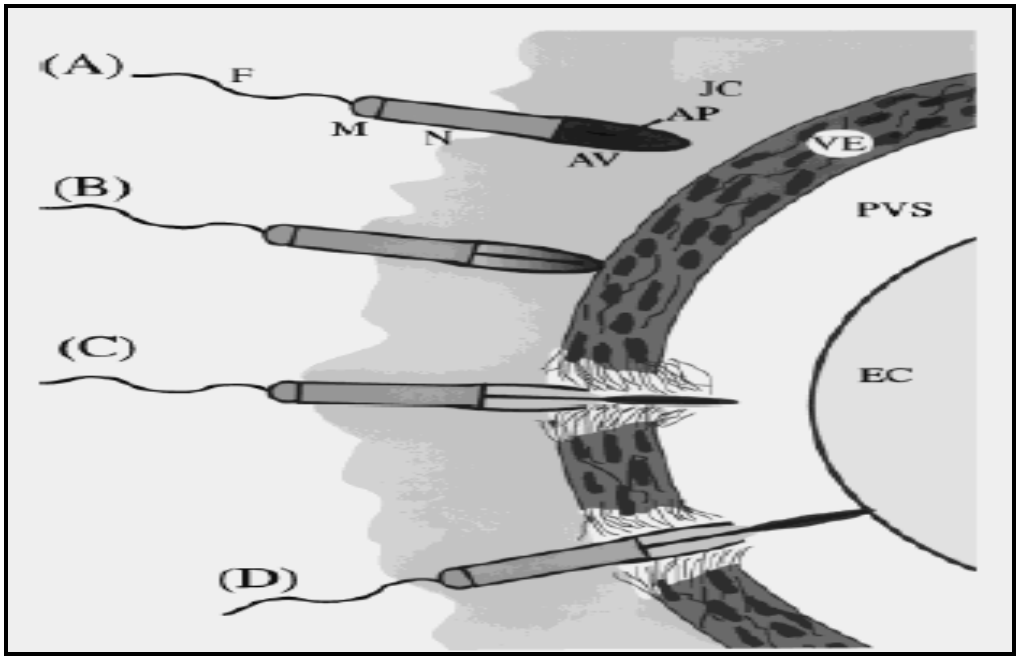

Figure 1.6 Sperm-egg interactions in the abalone (Kresge *et al*., 2001)

A: The sperm (F, flagellum; M, mitochondrion; N, nucleus; AP, acrosome process; AV, acrosomal vesicle) swims through the egg jelly coat (JC) and makes contact with the vitelline envelope (VE).

B: Contact initiates the acrosome reaction resulting in release of acrosomal proteins and the elongation of the acrosomal process.

C: The acrosomal proteins unravel the fibrous molecules and make a hole in the VE. The sperm passes through the hole into the perivitelline space (PVS).

D: The membrane covering the tip of the acrosomal process fuses with the egg plasma membrane and the sperm is drawn into the egg cytoplasm (EC).

1.2.3 Development

Abalone are ectotherms and therefore seawater temperature plays an important role in the fertilization success, timing of hatch-out and early life stages of abalone. Larval development occurs at a faster rate in warmer water, but is terminated at high temperatures, while sub-optimal temperatures result in a longer planktonic larval phase (Hahn, 1989; Henry, 1995). Haliotid larvae are considered to be lecithotrophic since they do not feed before settlement, but survive on nutrients in the egg yolk. Environmental food availability is therefore not a determining factor in the early development of larvae (Hahn, 1989; Henry, 1995).

Larval stages

Hahn (1989) distinguished 41 larval stages for *H. discus hannai* from fertilization until initiation of metamorphosis. The larval development stages of most abalone species are similar to that described by Hahn (1989) and summarized in Table 1.2.

Table 1.2 The larval development stages of *Haliotis discus hannai* from fertilization until initiation of metamorphosis as described by Hahn (1989)

1.	Fertilization
2.	Extrusion of first polar body (PB1)
3.	Extrusion of second polar body (PB2)
4-10.	Cleavage
11.	Cilia grow along the top of the embryo and starts beating
12.	Embryo rotates inside the egg membrane and stomodeum forms
13.	Cilia completely formed, embryo now called trochophore larva
14.	Egg membrane becomes thinner and finally bursts to hatch a trochophore larva approximately 0,2 mm in size (at this stage ± 14 - 22 hours has elapsed). The larvae can swim feebly at this stage (Fallu, 1991; Genade *et al.*, 1988; Hahn, 1989; Henry, 1995; McShane, 1992).
15.	Larval shell starts to be secreted on back of larva. Larva continues to develop until the veliger stage (approximately 24 - 48 hours has elapsed) (Genade *et al.*, 1988; Hahn, 1989; Henry, 1995; McShane, 1992).
16.	Larva now classified as veliger. Apical region becomes flat and the velum is completely developed with long cilia
17.	Larval retractor muscle forms
18.	Integumental attachment to larval shell forms
19.	Foot mass protrudes to top of shell
20.	Larval shell is completed
21.	Torsion starts: cephalo-pedal mass rotates 90˚ and top of mantle membrane tears off from top of larval shell
22.	Region destined to be mouth and foot rotate – until rotated 180˚
23.	Three long pairs of spines present at posterior end of metapodium after torsion
24.	Operculum forms

25.	Fine cilia develop on foot sole and begin beating
26.	Vertical groove forms in the velum
27.	Eye spots appear
28.	Propodium forms
29.	Cephalic tentacle forms on velum
30.	Cilia begin growing on propodium
31.	Cilia on propodium begin beating
32.	Propodium twists to side and apophysis appears
33.	Pair of epipodial tentacles form on both sides of foot under operculum
34.	Otolith forms and becomes clearly visible
35.	Short spines appear on cephalic tentacles
36.	Snout begins to protrude under velum
37.	Two tubules appear on cephalic tentacle
38.	Ciliary process forms on roof of mantle cavity
39.	Third tubule forms on cephalic tentacle
40.	Larval retractor muscle attached to shell draws enlarged mantle cavity towards back of shell
41.	Formation of fourth tubule on cephalic tentacle completes larval development. The veliger larva now shows crawling, exploratory movements characteristic of settling larvae (Hahn, 1989).

This whole developmental process takes four to ten days, depending on the species and water temperature (Hahn, 1989; Henry, 1995). The time that the larvae spend in a floating state is called the pelagic/planktonic stage. During this time the larvae change from trochophore to veliger stages. An investigation by Genade *et al*. (1988) confirmed that the planktonic larval stage of *H. midae* is more or less within the time confines of that of other abalone species.

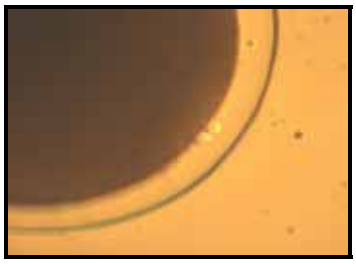

Figure 1.7 *H. midae* embryo arrested after extrusion of PB2 (De Beer, 2003)

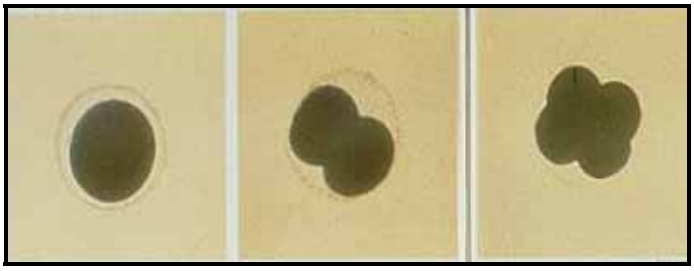

Figure 1.8 One, two and four cell stages in abalone larval development (Sorgeloos *et al.*, 1997)

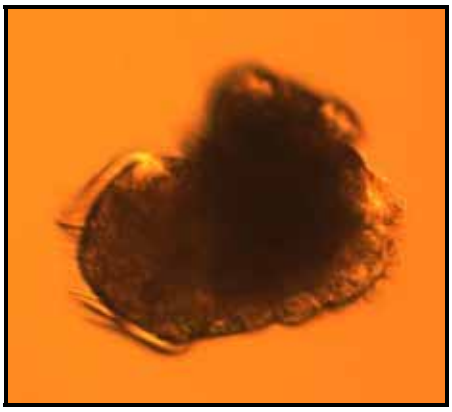

Figure 1.9 *H. midae* trochophore larva (24 hours) (De Beer, 2003)

Figure 1.10 Free-swimming (veliger) abalone larvae (Sorgeloos *et al.*, 1997)

Larval to post larval stages

Settlement, metamorphosis and deposition of the peristomal shell, characterize the transition from larval to post-larval development (Hahn, 1989).

Settlement occurs at a week to a month after the veliger stage, depending on the species and conditions. For *H. midae*, settlement occurs about five days at 20˚C and seven days at 17.5˚C after fertilization (Genade *et al.*, 1988). This is when the larvae sink to the bottom and start crawling in search of a suitable substratum. Crawling continues until the larvae attach to the substratum. Metamorphosis follows and is characterized by development of the mouth and radula, digestive tract, circulatory system with a heart beginning to beat, sensory organs and adult form (Hahn, 1989). Larvae are now called “spat” (or post-larvae) and feed on micro-algae (Fallu, 1991). Approximately 24 hours after metamorphosis they start feeding on benthic diatoms (Henry, 1995) and this will remain their principal food source until individuals are 7 to 10 mm in length, when they switch to eating macroalgae (Hahn, 1989).

Notch stage to sexual maturity

The post larval period continues until formation of the first respiratory pore, called the notch stage, which occurs at an age of about one to three months (Hahn, 1989). *H. midae* reaches the notch stage at a size of 2.1 - 2.2 mm and the first respiratory pore is completed at 2.3 mm (Genade *et al.*, 1988). Growth rates of juveniles sharply increase after the notch stage is reached as this is when weaning begins and the abalone starts feeding on macroalgae (seaweed) (Fallu, 1991; Hahn, 1989). Juveniles of about 10 mm in length consume 10 - 30 percent of their whole body wet weight in macroalgae each day. The abalone slowly increase in size until sexual maturity is reached (at around 7.2 years or 80 - 105 mm shell length in *H. midae*) and beyond (Barkai and Griffiths, 1988; Henry, 1995; Tarr, 1995).

1.2.4 Feeding behaviour and Energy metabolism

H. midae is entirely a herbivorous gastropod. The main source of energy of the adult abalone is kelp (*Ecklonia maxima*) which is ingested from late afternoon to early morning (Barkai and Griffiths, 1988). Large, mature individuals usually aggregate on an outcrop of reef, extending from 0.5 to 2 m above the seabed, facing the incoming swell and in the midst of dense kelp forests. Food availability in such aggregations is probably enhanced because of individuals trapping large drift-kelp fronds (Tarr, 1995). Food intake (wet weight) in wild *H. midae* is estimated at 8.1% of soft body weight per day at 14°C and 11.4% at 19°C (Sales and Britz, 2001) and the absorption efficiency of *H. midae* feeding on a natural diet of kelp is estimated at 37.25% (Barkai and Griffiths, 1988). Studies by Barkai and Griffiths (1988) on *H. midae* reported that about 63% of the energy consumed in food is lost as faeces in wild animals and a further 32% expended on respiration. It is suggested that some energy may also be lost to mucus production during locomotion, but this has not been confirmed (Barkai and Griffiths, 1988; Farias, Garcia-Esquivel and Viana, 2003). This leaves about 5% of energy intake available for growth and reproductive output with an increasing proportion of this energy utilized for reproduction in older animals. The assumption is made that energy expended on reproduction is similar for both male and female abalone (Barkai and Griffiths, 1988).

During the reproductive cycle, gametogenesis takes place. The production of gametes requires a large amount of nutrients for metabolic requirements and synthesis of vitellogenin, which serves as fuel for larval development (Hahn, 1989). The foot and the

digestive gland are indicated as sources of metabolic energy for gametogenesis. During gamete development, the size of the foot decreases and glycogen levels drop significantly. It is proposed that glycogen is converted to lipids and transferred to the ovary where it is incorporated in vitellogenesis. Additionally lipids are supplied to the ovaries by the digestive gland (Hahn, 1989). Consequently much metabolic energy is diverted towards gametogenesis and less energy and resources are available for somatic growth of the animal during reproduction (Boudry *et al.*, 1998; Garnier-Géré *et al*., 2002). As the abalone reaches sexual maturity, somatic growth is thus significantly reduced (Yang, Chen and Ting, 1998).

1.2.5 Farming of the South African abalone

The South African abalone fishery has been in existence since 1949, but the first attempts to cultivate *H. midae* were made in 1981 when Genade *et al* (1988) successfully spawned captured specimens to produce spat and juveniles (Genade *et al.,* 1988; Sales and Britz, 2001). The South African abalone, *H. midae* was, however, only identified as a suitable marine species for aquaculture in 1990 (Cook and Walmsley, 1990) and from then on concerted research and development efforts towards the establishment of commercial abalone farming began (Henry, 1995; Sales and Britz, 2001). Since then 12 abalone farms have been established, ranging in geographical distribution from Port Nolloth on the Atlantic/West Coast to East London on the Indian/East Coast (Sales and Britz, 2001). Today abalone farming in South Africa is entirely reliant on the culture of *H. midae* (Tarr, 1992), with commercial production increasing to 530 metric tonnes per year, with an estimated production value of R150 million per year, for 2003. The Western Cape is currently the region in South Africa where most of the aquaculture development is taking place, with abalone being one of the focus species (Brink, 2003).

Abalone has a very slow growth rate, typically two to three centimetres per year. At this rate, two to five years is required for an abalone to reach market size (Hahn, 1989). Like most other commercially important abalone species, the slow growth rate of *H. midae* is an obstacle in the profitable farming and global competitiveness of this species (Elliott, 2000; Stepto, 1997). Ongoing research in optimal culture conditions, nutrition and genetic improvement in abalone is addressing this problem of slow growth.

1.3 Triploidy

During sexual reproduction, germ cells undergo two maturation divisions through the process of meiosis before becoming gametes (see Figure 1.5). Meiosis starts with DNA replication and alignment of chromosome pairs to allow synapsis to take place. Halving of the chromosome number takes place at the end of Meiosis I when segregation of homologous chromosomes occurs and one chromosome from each homologous pair goes to each daughter cell. Meiosis II is essentially the same as mitosis where division of each chromosome into two daughter chromatids takes place. Sister chromatids of the same chromosome separate and become independent chromosomes that are drawn to opposite poles of the nucleus. Nuclear envelopes form around the chromosomes and the cytoplasm divides to form new cells, called gametes, with a haploid chromosome number. Male germ cells produce four gametes (sperm cells) during meiosis, while female germ cells produce only one gamete (ovum/egg). This is because the cytoplasm of female germ cells divides unequally. The products of the first maturation division are two cells with the same chromosomal constitution, but the one receives almost all of the cytoplasm. This cell is called the secondary oocyte. The other is called the first polar body. During Meiosis II the secondary oocyte undergoes the second maturation division and forms an ovum (egg) and another polar body, called the second polar body, which again has the same chromosomal constitution as the ovum, but very little cytoplasm. Neither of the polar bodies normally contributes any chromosomes to the final zygote which results from the fusion of sperm and egg and eventually the polar bodies are degraded (Beaumont and Fairbrother, 1991; Fairbanks and Anderson, 1999).

In marine molluscs, mature eggs are arrested at the prophase of Meiosis I and complete Meiosis I and II only after fertilization. Polar bodies 1 and 2 are thus released after fertilization and are in fact described as stages in larval development (Guo, Cooper, Hershberger *et al*., 1992; Hahn, 1989). This delayed meiosis provides a unique opportunity for manipulation of the polar bodies (Guo, Cooper, Hershberger *et al*., 1992).

Triploidy is a technology that has a lot of potential for application in commercial aquaculture because of its potential to induce sterility and therefore produce faster growing animals. Triploidy is induced in fertilized eggs during Meiosis I or Meiosis II by suppressing formation of either the first or the second polar body (Boudry *et al*., 1998).The result is that each cell nucleus contains one additional set of chromosomes

(contributed by the polar body) (Gérard *et al*., 1999). The resultant animal is termed a triploid. In adult triploid animals, the homologous chromosomes in the germ cells cannot synapse at meiosis and therefore normal meiosis cannot be completed and gametes cannot be produced (Beaumont and Fairbrother, 1991). Sterility is thus accomplished.

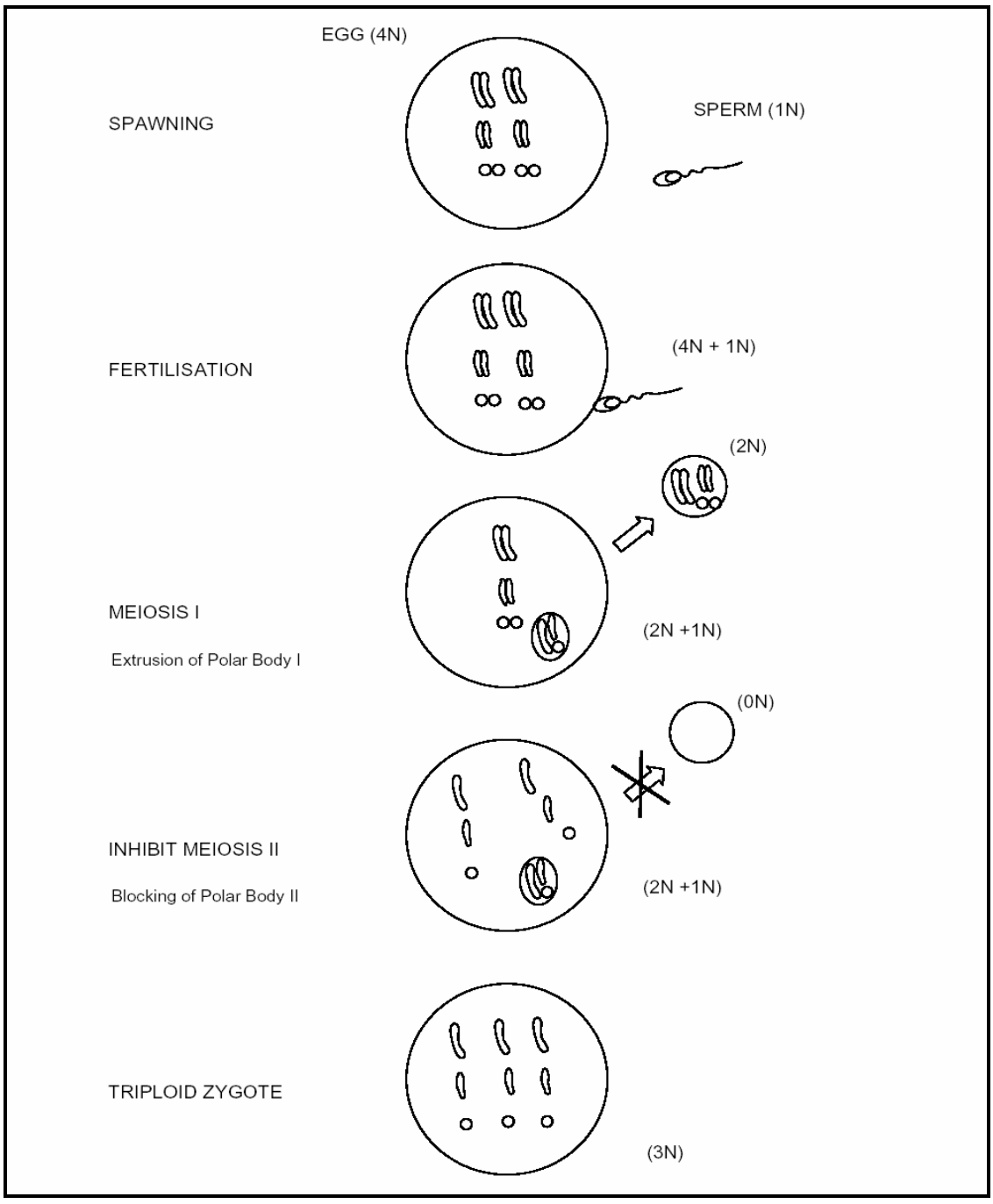

Figure 1.11 Sequence of events during triploid induction by inhibition of the second polar body in marine molluscs (Nell and Maguire, 1998)

1.3.1 Advantages of triploidy

Beaumont and Fairbrother (1991) supported by the authors indicated, highlighted three main reasons why sterility of aquaculture organisms is desirable from a commercial point of view:

1. Energy usually diverted to gamete production becomes available for somatic growth; therefore adult triploids should grow faster (Beaumont and Fairbrother, 1991).
2. The ripe gonad of adult animals in breeding season often render ripe animals unmarketable, while depleted glycogen levels during this period also negatively affects flavour. This disadvantage is eliminated in sterile animals (Beaumont and Fairbrother, 1991; Boudry *et al*., 1998; Chao, Yang, Tsai *et al*., 1993).
3. The risk of accidental introduction of non-native species from aquaculture facilities into the environment is reduced as such intruders will not be able to reproduce (Beaumont and Fairbrother, 1991; Kapuscinski, 2000).

Apart from the above, Yang, Chen and Ting (1998) also mention that because of larger nuclei in triploid cells, they can accommodate increased DNA and this leads to concomitant increase in overall cell size. On a genetic level, triploidy potentially induces higher mean heterozygosity because of the higher probability that triploids possess two or three different alleles per locus (Boudry *et al*., 1998; Garnier-Géré *et al*., 2002). This results in the phenomenon of “heterosis” or hybrid vigour which may be manifested as faster growth, higher viability or increased fitness (Beaumont and Fairbrother, 1991) as well as positively influencing feeding rate, absorption rate and growth efficiency (Garnier-Géré *et al*., 2002). Wang, Guo, Allen and Wang (2002) found a strong and positive correlation between meat weight and heterozygosity in triploid Pacific oysters. According to Magoulas, Kotoulas, Gérard, *et al.* (2000), triploids perform better than diploids because of a potential for faster transcription due to the presence of three copies of genes instead of two. Hawkins and Day (1996) proposed that triploids may have greater stress resistance than diploids.

One negative aspect that can be associated with induced triploidy, namely reversion of triploids to heteroploid mosaics containing diploid and triploid cells, was pointed out by Allen and Guo (1996). After a season of disease challenge in the field, about 20 percent of supposed triploid oysters were heteroploid mosaics which may have arisen as a consequence of chromosome loss. The possibility of heteroploid mosaics producing

gametes was investigated, but no evidence of haploid gamete production has been found. Although triploids can occasionally produce gametes that are fully capable of fertilization, aneuploid progeny results from such fertilizations and it was concluded that the reproductive potential of triploid oysters is extremely low (Guo and Allen, 1994a). The phenomenon that triploids are unstable over time might suggest that the reproductive potential does not stay as low as in the original pure triploid population, but this has not been confirmed (Allen and Guo, 1996).

Induction of triploidy can be achieved through the prevention of extrusion of either the 1st polar body from Meiosis I or that of the 2nd polar body from Meiosis II. Much controversy still prevails about which of the two inhibitions is more successful. Meiosis I triploids are suggested to be more heterozygous and therefore exhibits faster growth rates than Meiosis II triploids (Guo, Cooper, Hershberger *et al*, 1992; Guo, Hershberger, Cooper *et al.,*1992*;* Beaumont and Fairbrother, 1991). However, the blocking of polar body 1 results in higher mortalities than the blocking of polar body 2 (Guo, Cooper, Hershberger *et al*, 1992; Guo, Hershberger, Cooper *et al.,*1992). Meiosis I triploids may also be more prone to abnormal development and aneuploidy than Meiosis II triploids (Gérard *et al*., 1999). For most species, the blocking of polar body 2 is still used as the preferred method for inducing triploidy (Liu, Heasman and Simpson, 2004; Maldonado, Ibarra, Ramirez, Avila, Vazquez and Badillo, 2001).

1.3.2 Methods for inducing triploidy

The different methods that can be used to induce triploidy include: (i) chemical shock (cytochalasin B, 6-dimethylaminopurine, calcium or caffeine), (ii) thermal shock (heat or cold shocks), (iii) pressure shock, (iv) electrical field shock or (v) combinations of these (Beaumont and Fairbrother, 1991; Scarpa, Toro and Wada, 1994; Stepto, 1997; Stepto and Cook, 1998).

Chemical treatment

One of the most commonly used methods of triploidy induction is chemical shock by means of Cytochalasin B (CB) treatment. CB is a fungal metabolite (produced by the fungus *Helminthosporium dematioideum*) that is thought to inhibit micro-filament formation in cells by inhibiting actin polymerization (Beaumont and Fairbrother, 1991; Gérard *et al*., 1999). CB treated cells do not show the presence of a cytoplasmic cleavage furrow or filaments of the contractile ring needed for effective cytokinesis

(division of cytoplasm during meiosis) (Beaumont and Fairbrother, 1991). Induction by means of CB treatment requires less critical timing of treatment and generally produces higher percentages of triploidy than other treatments (Beaumont and Fairbrother, 1991). It has been used with success in the Pacific red abalone, *H. rufescens* (Maldonado *et al*., 2001), the Australian blacklip abalone, *H. rubra* (Liu *et al*., 2004), the Pacific abalone, *H. discus hannai*, (Wang *et al*., 1990), the small abalone, *H. diversicolor supertaxa* (Yang, Ting and Chen, 1998), the South African abalone, *H. midae* (Stepto, 1997, Stepto and Cook, 1998) and various oyster species (Gérard *et al*., 1999; Barber, Mann and Allen, 1992). However, this treatment is expensive and tends to produce ploidy levels higher than the required triploidy, due to increased polyspermy as well as higher percentages of abnormalities and increased mortality during early larval development when compared to other methods (Beaumont and Fairbrother, 1991; Stepto, 1997). Additional concerns have been expressed with regard to the health hazard to humans associated with high cytotoxicity and carcinogenic activity of CB, even though its use on a commercial basis has been approved in the USA (Beaumont and Fairbrother, 1991; Liu *et al*., 2004).

A safer chemical that has shown to inhibit polar body extrusion in marine molluscs is the puromycin analogue, 6-dimethylaminopurine (6-DMAP) (Liu *et al*., 2004; Norris and Preston, 2003). Species where triploid induction was successfully applied with 6-DMAP include the Tropical abalone, *H. asinina* (Norris and Preston, 2003), the Pacific abalone, *H. discus hannai* (Zhang, Wang, Chang, Song, Ding, Wang and Wang, 1998), the Australia blacklip abalone, *H. rubra* (Liu *et al*., 2004), the Pacific oyster, *Crassostrea gigas* (Gérard *et al*., 1999) and the Sydney rock oyster, *Saccostrea commercialis* (Nell, Hand, Goard, McAdam and Maguire, 1996). Limited success has also been reported for chemical shock treatments such as calcium and caffeine treatment. Calcium treatment did not result in efficient triploidy induction, while both calcium and caffeine are associated with poor larval development and survival (Scarpa *et al*., 1994).

Thermal treatment

The physical methods of triploidy induction include temperature shock and hydrostatic pressure shock. These methods are thought to interfere with normal meiotic and mitotic cell division and normal development resumes, after temperature is normalized or pressure is released (Beaumont and Fairbrother, 1991). Temperature shock has been used slightly more in for example the small abalone, *H. diversicolor*, with cold shock (Yang, Chen and Ting, 1998), the Pacific abalone, *H. discus hannai*, with heat and cold

shock (Arai *et al.,* 1986), the South African abalone, *H. midae*, with heat shock (Stepto, 1997), the Pacific oyster, *Crassostrea gigas*, with heat shock (Quillet and Panelay, 1986) and the mussel, *Myelitus edulis,* with heat and cold shock (Yamamoto and Sugawara, 1988).

Pressure treatment

Triploid induction by means of hydrostatic pressure treatment has been documented for the Pacific oyster, *Crassostrea gigas* (Chaiton and Allen, 1985; Allen, Downing, Chaiton and Beattie, 1986), the Pearl oyster, *Pinctada martensii* (Shen *et al.*, 1993) and the Pacific abalone, *H. discus hannai* (Arai *et al.,* 1986; Curatolo and Wilkins, 1995), but is not nearly as common as the chemical induction methods.

The limitation of physical induction methods (thermal and pressure) is that these treatments arrest all development and therefore only eggs which are at a vulnerable stage of cell division at the time of shock is affected by the treatment (Beaumont and Fairbrother, 1991; Griffiths, 1994). Additionally, pressure shock requires a specially manufactured pressure vessel, capable of safely withstanding very high pressures (Beaumont and Fairbrother, 1991).

Electrical field treatment

Limited success has also been reported with electrical field shock treatment. Electrical field shock requires extremely specialized and complex procedures. Zygotes require specific orientation and need to be individually manipulated, making this a very time consuming induction method (Cadoret, 1992).

Use of tetraploids

The limitations associated with the use of chemicals and physical induction methods may be overcome by the crossing of tetraploids and diploids, producing 100 percent triploid progeny (Allen and Guo, 1996). Guo and Allen (1994 a,b) was the first to succeed in producing tetraploid Pacific oysters using eggs from triploid Pacific oysters (which can on occasion reach fecundity with gametes fully capable of fertilization) in which they blocked the first polar body with CB treatment. Since then, they have shown the general usefulness of tetraploids for making 100%-triploid populations (Guo, DeBrosse and Allen, 1996). Eudeline, Allen and Guo (2000) developed a technique for producing tetraploids that relies on biological criteria of individual females rather than on general rules applicable to all females. They recommended to start treatment at the first signs of

appearance of polar body 1 and ending it when 50% polar body 1 are visible using a single female at a time. Their results demonstrated that this approach enables a significant improvement over previous techniques. Wang *et al.* (2002) found that triploid gigantism is better expressed in triploids from tetraploid-diploid crosses than in triploids produced by blocking polar body 2 with cytochalasin B because of further increases in heterozygosity or other genetic factors. It has been suggested that the mating of tetraploids and diploids is the best method for triploid production, and triploids produced in this way are better suited for aquaculture than those produced by altering meiosis (Guo *et al.,* 1996). Mating of tetraploids and diploids is thus being incorporated in the production of triploids on an increasing scale.

1.4 Identification of triploidy

A reliable method of assessing the levels of triploidy in treatment groups is of utmost importance to ensure that time and space will not be wasted rearing batches of larvae with low triploid yields. It is also important to keep track of changes of triploid percentages over time, this being a common phenomenon probably due to differential mortality between triploids and diploids (Beaumont and Fairbrother, 1991; Liu *et al*., 2004). Different methods can be employed to determine triploidy percentages in treated groups.

1.4.1 Chromosome analysis/Karyotypic analysis

Chromosomes analysis entails direct counting of chromosomes under high magnification. It is the most direct indication of the presence of triploidy, where triploid samples contain 1.5 times the number of chromosomes of diploids. Karyological analysis is viewed as an unequivocal indicator of ploidy, but it is the most laborious and time consuming method (Chaiton and Allen, 1985; Chao, Hsu, Hsu, Liang and Liao, 1993). Direct chromosome counts to determine triploidy levels can be done readily at day 0 and at the trochophore larval stage (Nell, 2002; Nell *et al*., 1996). More complex procedures can also be implemented using tissue from spat, juveniles and adults (Beaumont and Fairbrother, 1991). The procedure usually involves arresting cells in metaphase for chromosome counting. Embryos/larvae are prepared by exposing them to 0.01% colchicine, which arrest cells in the metaphase of mitosis, before they are transferred to a hypotonic solution of sodium citrate or potassium chloride. Embryos/larvae are then fixated in Carnoy's fixative (methanol and glacial ascetic acid). A suspension of the fixed larvae is dropped onto a glass slide, air-dried and stained with Giemsa solution. Cell counts of metaphase chromosomes are taken under a microscope (600 x or higher magnification) (Chao, Hsu, Hsu *et al*., 1993; Nell *et al*., 1996). Allowance should be made for chromosome loss and overlapping of cells during slide preparation. Therefore, a range of chromosome numbers is used to give a realistic estimate of ploidy rather than a strict application of chromosome number (Guo, Cooper, Hershberger *et al*., 1992; Nell, 2002; Nell *et al*., 1996).

Chromosome analysis have been used with success for ploidy determination in many shellfish species, including the Pacific abalone, *H. discus hannai* (Arai *et al*., 1986), the Small abalone, *H. diversicolor* (Yang, Chen and Ting, 1998; Yang Ting and Chen, 1998), the Pacific oyster, *Crassostrea gigas* (Guo, Cooper, Hershberger *et al*., 1992), the

Sydney rock oyster, *Saccostrea commercialis* (Nell *et al*., 1996) and the Zhikong Scallop, *Chlamys farreri* (Yang, Zhang and Guo, 2000).

1.4.2 Nuclear sizing

Nuclear sizing involves comparison of the nuclear size/volume of cells between diploids and triploids (Nell, 2002). The size of the nucleus reflects the degree of ploidy because it contains the chromosomal material. The nuclei of triploid cells are 1.5 times the volume of diploid cells' nuclei and will therefore have a greater diameter (Beaumont and Fairbrother, 1991; Chao, Hsu, Hsu *et al*., 1993).

Red blood cells (erythrocytes) are usually used to determine ploidy in fish (Chao, Hsu, Hsu *et al.*, 1993; Rottmann, Shireman and Chapman, 1991). Blood smears are made on a slide and the minor and major axis of the nucleus is measured directly using an eyepiece micrometer (Chao, Hsu, Hsu *et al*., 1993). Studies by Child and Watkins (1994) on the Manila clam, supported by studies by Nell (2002) on oysters demonstrated that measuring of the diameter of cell nuclei from gill tissue and hemolymph also distinguished successfully between diploids and triploids. An improved method for estimating ploidy electronically, using nuclear sizing, is the Coulter Counter, which can be calibrated to read both diploid and triploid red blood cell nuclei volumes (Chao, Hsu, Hsu *et al*., 1993; Chao, Yang, Tsai *et al*., 1993; Rottmann *et al*., 1991).

Nuclear sizing is considered a simple technique with the advantages that it can be carried out using a high power microscope and basic microbiological equipment. It is also a cheap and easy method of determining ploidy (Child and Watkins, 1994). In a comparison of triploid induction validation techniques, Harrell, van Heukelem and Kerby (1995) concluded that nuclear sizing (particle size analysis) is the simplest and quickest method for evaluating ploidy in fish.

1.4.3 Microfluorometry

This method uses slides made from cell suspensions that are stained with the Feulgen reaction procedure or a fluorescing DNA specific dye, like 4'-6-diamidino-2-phenylindole (DAPI). DAPI binds preferentially to the adenine-thymine base pairs of DNA which is then excited with ultra-violet light. The fluorescence intensity is measured by a photometer. A high quality ultra-violet microscope with a photometer is therefore a requirement for this technique (Beaumont and Fairbrother, 1991; Griffiths, 1994). Readings of stains vary with

DNA content in the cell, with triploids having 1.5 times the reading of the control (diploids) (Chao, Hsu, Hsu *et al*., 1993; Chao, Yang, Tsai *et al*., 1993).

Chao, Yang, Tsai *et al*. (1993) used microfluorometry to determine ploidy of blood smears of the cyprinid loach, *Misgurnus anguillicaudatus* and the common carp, *Cyprinus carpio*. Scarpa,Toro and Wada (1994) used the same technique for estimating triploidy by measuring DNA content in cells from trochophore larvae of the blue mussel, *Mytilus galloprovincialis.* In the scallop, *Chlamys nobilis*, cells from gill tissue and hemolymph were used to measure triploidy by DNA microfluorometry with DAPI staining (Komaru, Uchimura, Ieyama and Wada, 1988). Microfluorometry was also successfully used in detecting induced triploidy in the larvae of the Japanese pearl oyster, *Pinctada fucata martensii,* with DAPI staining (Uchimura, Komaru, Wada, Ieyama, Yamaki and Furuta, 1989).

1.4.4 Image analysis

Image analysis measures the optical density of stained nuclei (Nell, 2002). Gérard, Naciri, Peignon, Ledu, Phelipot, Noiret, Peudenier and Grizel (1994) proposed this method as an easy, efficient alternative for karyological determination (chromosome analysis), microfluorometry and flow cytometry. Larvae/tissue from the oysters *Crassostrea gigas*, and *Ostrea edulis* and the clam *Ruditapes philippinarum* were prepared by fixing them on slides and staining with the standard Feulgen-Rosalin method. Slides were examined under a microscope, connected to a computer. A specialized program called *Samba™ 2005* was used to analyze the photometric intensity of stained nuclei and reported their individual integrated optical density (IOD). DNA indices are computed from the IOD values and are expected to be 1.0 for diploids and 1.5 for triploids. Image analysis can be used for ploidy determination in embryo, larvae, juvenile and adult preparations (Gérard *et al*., 1994).

1.4.5 Flow Cytometry

Flow cytometry, according to Ormerod (1999), is the measurement of cells in a flow system that has been designed to deliver particles in single file past a point of measurement. Flow cytometry has various applications, including DNA analysis. When used for ploidy analysis, flow cytometry measures the fluorescence of the cell nucleus after it has been stained with a DNA intercalating fluorescent dye, like propidium iodide

(PI), Ethidium Bromide, Acridine Orange or DAPI (Allen, 1983; Chaiton and Allen, 1985; Liu *et al*, 2004). The amount of dye taken up by the cell is generally proportional to the amount of DNA in the nucleus. Stained nuclei of triploid cells will therefore emit 1.5 times the fluorescence of diploid nuclei (Nell, 2002). This technique enables the researcher to gather information about tens of thousands of cells/nuclei within a few minutes (Ormerod, 1999). Flow cytometry is a powerful method for ploidy determination and has been used extensively on triploid induced shellfish such as the Australian blacklip abalone, *H. rubra* (Liu *et al*., 2004), the Pacific red abalone, *H. rufescens* (Maldonado *et al.*, 2001), the Tropical abalone, *H. asinina* (Norris and Preston, 2003), the South African abalone, *H. midae* (Stepto, 1997; Stepto and Cook, 1998) and various oyster species (Allen *et al.*, 1986; Supan, Wilson and Allen, 2000).

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CHAPTER 2: The use of Flow Cytometry for the evaluation of ploidy in the South African abalone

2.1 Introduction

Flow cytometry, “the measurement of cells in a flow system that has been designed to deliver particles in single file past a point of measurement” according to Ormerod (1999) is defined by Shapiro (1994) as “the measurement of physical and/or chemical characteristics of cells or, by extension, of other biological properties. Flow cytometry is a process in which such measurements are made while the cells or particles pass, preferably in single file, through the measuring apparatus in a fluid stream”. The point of measurement is light focused on particles, recording their fluorescence and the light scattered by them. This technique enables the researcher to gather information about tens of thousands of cells/particles within a few minutes. The data so generated are processed with a well programmed computer into histograms (Ormerod, 1999).

One of the first flow cytometers was built by Mack Fulwyler in the early 1960’s at Los Alamos, but the development of flow cytometers for clinical research was made possible by Louis Kamentsky. In the late 1970’s, the development of commercial flow cytometers became economically feasible, with the first machine with production potential being developed by Herzenberg’s group at Stanford (Mandy, Bergeron and Minkus, 1995).

Applications of Flow Cytometry

Application of flow cytometry in the clinical field showed tremendous growth over the past two decades and today the three general fields where flow cytometry technology is well established are clinical immunology, laboratory haematology and medical oncology (Mandy *et al*., 1995; Ormerod, 1999). A variety of cell properties may be measured, for example, DNA content of nuclei, expression of a surface antigen or activity of an intracellular enzyme. Other applications of flow cytometry include: cell proliferation studies, apoptosis studies, determination of RNA content, protein content, membrane permeability and membrane potential, measurement of drug uptake, binding and endocytosis of ligands, measurement of intracellular pH, chromosome analysis and sorting and measurement of cell viability. The use of flow cytometry in all these and many more fields, testifies of its versatility and improvement over the past 25 years (Ormerod, 1999; Vindeløv and Christensen, 1994).

The measurement of DNA is the second most common application of flow cytometry, after measurement of surface antigens by immunofluorescence (Ormerod, 1999). Measurement of the DNA content of a cell gives a representation of the cell cycle. Ploidy measurements can be used for determining the ploidy of tumours where an estimation of the S phase fraction gives an indication of the proliferative state of a tumour (Ormerod, 1994).

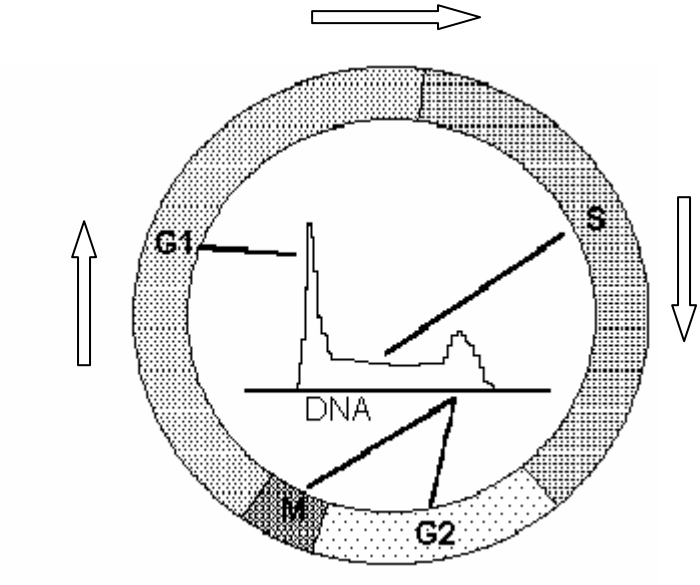

Figure 2.1 Relationship between the cell cycle and a DNA histogram acquired by flow cytometry (Ormerod, 1994)

Quiescent cells, that are not growing or dividing, are referred to as being in the G_0 state. As a somatic cell prepares to divide, it enters the G_1 phase of the cell cycle during which RNA increases and proteins essential for DNA replication are made. At the end of G_1, the S-phase (synthesis phase) is initiated when DNA replication begins. This is when the DNA content of the cell increases until it has doubled. DNA synthesis then ceases and the cell enters the G_2 phase of the cell cycle. Finally the cell enters mitosis where the DNA is halved and the cell divides to form two new daughter cells. Most somatic cells are arrested at the G_1 phase (Fairbanks and Anderson, 1999; Ormerod, 2000).

In this study, as in the case of clinical tumour sample analysis, flow cytometry is used to measure ploidy. The use of flow cytometry for the assessment of ploidy in shellfish was first reported by Allen (1983) when he used a Ortho diagnostic Systems Cytofluorograph to assay granulocytes and hemolymph from diploid and triploid shellfish (oysters and

clams). Flow cytometry was found to be a fast, accurate procedure for evaluating polyploidy in shellfish which can be applied to a variety of tissues without sacrificing the animal. Such tissues include hemolymph, gill, mantle, siphon and foot tissue as well as larvae and spat (Allen, 1983; Beaumont and Fairbrother, 1991; Chaiton and Allen, 1985; Yang, Gallivan, Guo and Allen, 2000). It is now the most popular way of identifying triploidy in shellfish.

Description of a basic flow cytometer system (see Figure 2.2)

During flow cytometry, measurements are made separately on each particle in the suspension. Multiple cellular parameters can be measured, based on light scatter and fluorescence. Particles are delivered singly to the point of measurement by injection into the centre of an enclosed channel through which the liquid flows. Within such a channel/flow chamber the sample stream, surrounded by sheath fluid, is hydrodynamically focused to be delivered to the point of measurement with an accuracy of about 1 µm (Carter and Ormerod, 2000, Ormerod, 1999). Photodetectors, called photomultiplier tubes, collect the scattered and fluorescent light which is generated by cells passing through the illuminating beam. To attain specific independent, but correlated measurements from stained particles, the scattered and fluorescent light need to be separated. Separation of different wavelengths is achieved through the use of dichroic mirrors and interference and absorption filters (Carter and Ormerod, 2000). Mirrors, filters and detectors can be added and adjusted to meet the needs of different samples (The Wistar Institute Flow Cytometry Facility, 2004).

Light falling on the photodetector surface generates a current that is fed into amplifiers. A system threshold can be set so that further processing only takes place when the input voltage rises above a pre-set value, thus preventing background noise. An analog-to-digital converter now converts the information into electronic signals so that it can be presented in binary code. The computer processes this information into meaningful data which result in graphic display (like frequency histograms or dual parameter correlated plots) and statistical analysis of the measured parameters (Carter and Ormerod, 2000).

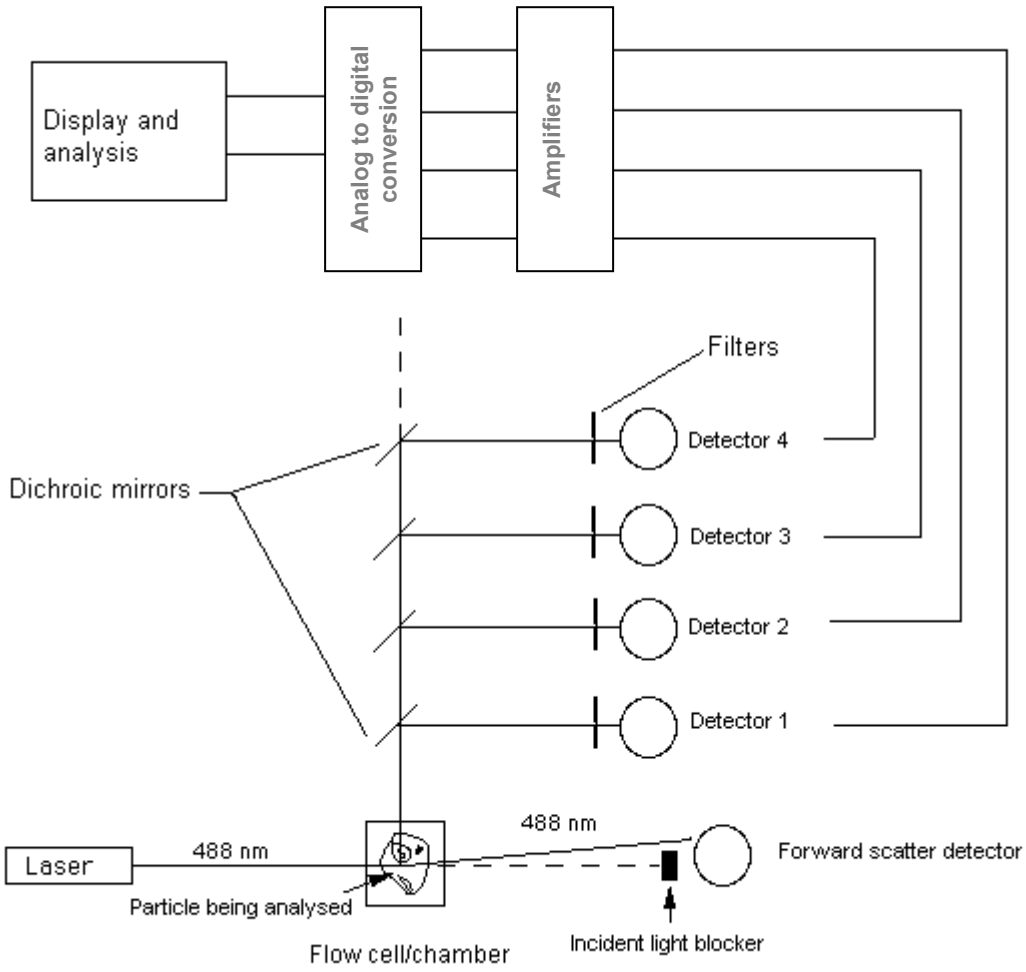

Figure 2.2 Generalized basic flow cytometer system (Carter and Ormerod, 2000; The Wistar Institute Flow Cytometry Facility, 2004)

The forward scatter detector and right angle scatter detector (detector 1 in Figure 2.2) distinguish cells based on their morphology (forward scatter ~ particle size; right angle scatter ~ particle granularity), while the fluorescent detectors measure the amount of flourochrome binding (The Wistar Institute Flow Cytometry Facility, 2004).

The optical system of the flow cytometer is designed to measure illuminated cells (Mandy *et al*., 1995). In order for detectors to measure fluorescence, the particles need to be stained with a fluorescent dye. For measuring ploidy a variety of fluorescent dyes are available that bind stoichiometrically to DNA. Examples are DAPI (4,6-diamidine-2-phenylindole), Ethidium Bromide, Acridine Orange and Hoechst 33342 (Ormerod, 1999; Vindeløv and Christensen, 1994; Yang *et al*., 2000). Due to the hydrophobisity of the environment, these dyes fluoresce strongly when bound to DNA (Ormerod, 1999). A

widely used dye, and the one that was used in this study, is the flourochrome, Propidium Iodide (PI). PI is a red powder, with molecular formula $C_{27}H_{34}N_4I_2$ (Sigma Product Information, 2003). It binds specifically to double-stranded nucleic acids by intercalation between base pairs, without base specificity. PI absorbs in blue-green (493 nm), fluorescing red (630 nm) and has an absorption spectrum with maxima at 287 and 488 nm. PI readily enters and stains non-viable cells, but cannot cross the membrane of viable cells. In order to stain the nuclei, viable cells must first be treated to permeabilize their membranes/ isolate their nuclei. It also binds to double stranded RNA which should therefore be removed with ribonuclease (RNase) before staining (Ormerod, 1999; Sigma Product Information, 2003; Vindeløv and Christensen, 1994).

Ploidy determination in *Haliotis midae*

When determining ploidy, the technique assumes that fluorescence is proportional to ploidy. The differences in fluorescence caused by the different ploidy levels are big enough to be unambiguously determined (Griffiths, 1994). In this study, the DNA content of nuclei from abalone larval cells, stained with propidium iodide, was measured to discriminate between diploid and triploid larvae. The resulting graphics describe the distribution of fluorescence signals from the nuclei by frequency distribution histograms (Yang *et al*., 2000).

Flow cytometry has been used once before for ploidy analysis in the South African abalone, *H. midae*, when Stepto (1997) assayed the ploidy of chemically and temperature induced triploidy in abalone larvae. The aim of the present study is to evaluate flow cytometry as a method of identification of triploidy in hydrostatic pressure induced larvae from the South African abalone, *H. midae*.

2.2 Materials and Methods

The material that was used for flow cytometry consisted of larvae from the South African abalone, *H. midae* that received hydrostatic pressure treatment in order to induce triploidy. The eggs were subjected to hydrostatic pressure shortly after fertilization. Treated eggs were hatched and the larvae collected 48 hours post fertilization and frozen in Vindeløv citrate buffer (see Appendix 2) with 10% DMSO at -20˚C. For preliminary studies, abalone spermatozoa and normal, diploid abalone larvae, preserved in the same way at -20˚C, were used.

2.2.1 Nuclear isolation

The three different methods that were examined for the isolation of nuclei are discussed below:

Method 1: Vindeløv

The first method (Vindeløv and Christensen, 1994) consists of three steps: In the first step, clean nuclei are obtained by the combined action of a detergent and trypsin. Spermine and trypsin inhibitor are used to ensure stability of the nuclei during and after trypsinization. Double stranded RNA is removed by digestion with ribonuclease (RNase) which is added during the second step. The dye that is used, Propidium Iodide (PI), binds to double stranded nucleic acids by intercalation, and is added in the final step.

For nuclear isolation using the Vindeløv method, normal embryo larvae and sperm were thawed at 37˚C in a water bath. Samples of 200 µl were taken and 1.8 ml of Solution A (see Appendix 2) added to each. The samples were left at room temperature for ten minutes, after which 1.5 ml of Solution B (see Appendix 2) was added. After ten more minutes at room temperature, 1.5 ml of ice cold Solution C (see Appendix 2) was added. The samples were filtered through 25 µm nytex mesh, wrapped in foil and kept on ice (Vindeløv and Christensen, 1994). After approximately 20 minutes, the samples were examined under a fluorescence microscope and subsequently analyzed on the flow cytometer.

Method 2: PI staining solution (sperm)

Sperm samples were taken from the -20˚C freezer and thawed at room temperature. From each sample, 50 µl of sperm was taken and 50 µl MPBS (Allen, 1983; see Appendix 2) and 500 µl Propidium iodide (PI) staining solution (Norris and Preston, 2003;

see Appendix 2) was added to it. The suspension was then filtered through a 25 µm nytex screen twice (Allen, Gaffney, Scarpa and Bushek, 1993) before the samples were wrapped in foil and kept on ice until examined under the fluorescence microscope and subsequent analysis by flow cytometry.

Method 3: PI staining solution and aspiration (larvae)

Larval samples were taken from the -20˚C freezer and thawed at room temperature. From each sample, 50 µl of concentrated larvae was taken and 50 µl MPBS (Allen, 1983; see Appendix 2) and 500 µl Propidium iodide (PI) staining solution (Norris and Preston, 2003; see Appendix 2) was added to it. The suspension was then aspirated through a 1 ml syringe fitted with a 26½ G needle (Allen *et al*., 1993) until no more clogging of the needle's tip occurred. The cell suspension was then filtered through a 25 µm nytex screen twice (Allen *et al.,* 1993) and centrifuged for 5 minutes at 5000 rpm. The supernatant was removed and the pellet of stained larval nuclei resuspended in 500 µl MPBS in a plastic eppendorf tube. The tube was wrapped in foil and kept on ice until examined under the fluorescence microscope and subsequent analysis by flow cytometry.

2.2.2 Fluorescence microscopy

Microscopic review of the stained nuclei is an essential control of the process of DNA flow cytometry, since it gives information regarding the uptake of dye into the nucleus, integrity of nuclei and the degree of aggregation/clumping of nuclei (Dressler, 1990). If excessive aggregation of nuclei and debris occur, or no stained nuclei are visible, flow cytometry of the concerned sample will be unsuccessful. Microscopic screening of fluorescent samples may also be helpful in the interpretation of difficult DNA histograms (Dressler, 1990). Two cells in the G_1 phase of the cell cycle which is stuck together will have the same DNA content as a cell in the G_2 phase (Ormerod, 1994) and an incorrect representation of the true DNA status of the sample will be conveyed in the histogram. Therefore it is important that only single cells should be recorded when analysing a DNA histogram (Ormerod, 1994). The prepared samples were examined under 10 times and 20 times magnification on a Nikon Eclipse E400 microscope. Samples were examined for clumps of nuclei and purity of the sample. Pictures were taken with a Nikon Digital still camera DXM 1200 mounted on the microscope by making use of the software Nikon ACT-1 Version 2.1.2 (Nikon Corporation).

2.2.3 Flow cytometry

The successfully prepared samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer with a He/Ne laser, with wavelength 488 nm (Figure 3.8). It is a bench-top instrument with a cuvette flow-cell, where the cells are singly delivered to a specific point where the laser is focused. The machine is linked to a Mackintosh computer from where the software runs. Prior to running, samples were vortexed to obtain a suspension of nuclei and transferred to a 5 ml Becton Dickinson flow cytometry tube. For each sample, a minimum of 20 000 nuclei were acquired at a rate of 100 to 250 nuclei per second to generate histograms. The following instrument settings were used to attain adequate histograms. Instrument settings are adjusted and acquisition is carried out via the specialized software, BD CellQuest Pro^{TM} (Becton Dickinson).

Table 2.1 Instrument settings for the Becton Dickinson Flow Cytometer via the program CellQuest ProTM used to analyze larvae of the South African abalone induced for triploidy with the hydrostatic pressure method

	Volt	**Amp**	**Mode**
P1	E00	3.00	lin
P2	500	1.00	lin
P3	320	1.00	log
P4	490	1.00	lin
P5	380	1.00	log
P6	-	1.00	lin
P7	-	3.94	lin
Threshold	20		
Compensation	0		
P = Photomultiplier detectors (P1: Forward angle light scatter; P2: Right angle light scatter; P4: Fluorescent label 2 PI-DNA; P6: Fluorescent label 2 Area; P7: Fluorescent label 2 Width).			

The fluorescence intensity of stained nuclei are recorded and digitally converted from the flow cytometer to the computer. Threshold is set to ignore debris and minimize electronic noise. DNA fluorescence was used as the thresholding parameter.

2.2.4 Software analysis of raw data

The raw data were analysed using specialized software programs. The programs Modfit LT (Verity Software House, Topsham, ME, USA) and WinMDI Version 2.1.3 (Copyright© 1993–1996, Joseph Trotter) were used for analysis. Automatic analysis was mainly done, with occasional manual analysis to provide histograms. These histograms describe the distribution of fluorescence signals from the nuclei (Yang *et al*., 2000). The peak position of the histogram is measured by channel numbers on the horizontal axis and reflects the relative DNA content per nucleus, while the number of nuclei recorded is shown on the vertical axis (Peruzzi and Chatain, 2000; Yang *et al*., 2000). The area under the curve represents the relative contribution of a certain ploidy (Allen, 1983).

2.3 Results and Discussion

The results obtained from sample preparation and flow cytometry are presented in the form of pictures (Figures 2.3 – 2.4) and graphs (Figures 2.5 – 2.10).

The goal of sample preparation is to obtain single nuclei with minimum degradation of the DNA and minimum clumping/aggregation and debris. It is important for subsequent analysis of the DNA histogram that only single cells should be recorded by the flow cytometer (Ormerod, 1994). Clumping/aggregation occur when two or more cells are stuck together as a result of the sample preparation method or the method of fixation (especially when 70% ethanol is used). Clumping is indicated as a green coloured area in DNA histograms generated by Modfit LT. Debris consists of bacteria, impurities and small cellular pieces resulting from harsh degradation of the larval cells. Debris is represented by a purple coloured area in DNA histograms generated by Modfit LT.

Before running samples on the flow cytometer, microscopic examination is carried out to verify if samples are adequately prepared for flow cytometry i.e. with minimum clumping and debris. Figures 2.3 and 2.4 are examples of adequately and inadequately prepared samples of PI-stained larval nuclei respectively.

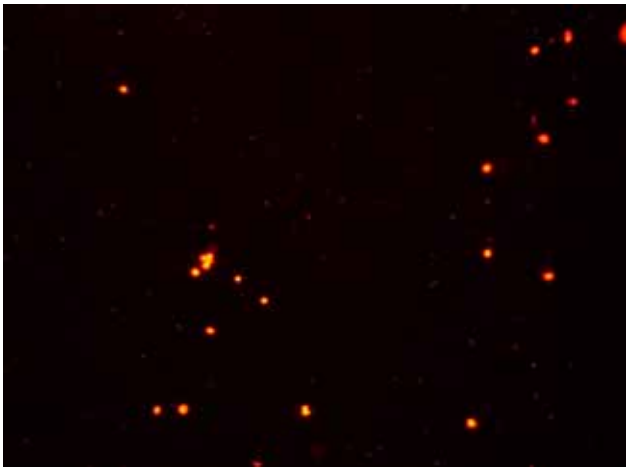

Figure 2.3 Example of proper sample preparation of a pure sample of PI-stained abalone larvae nuclei prepared for flow cytometry (De Beer, 2003)

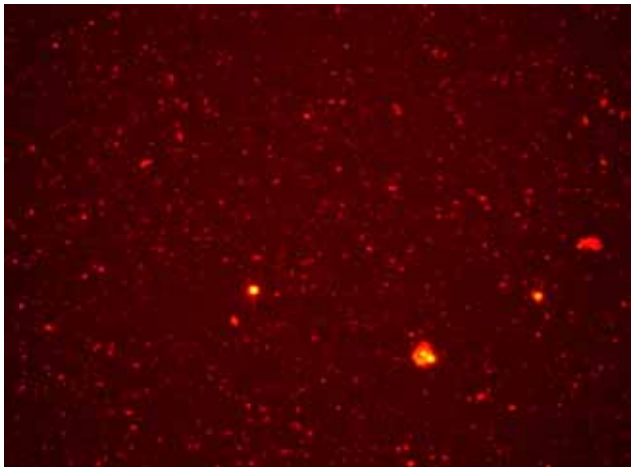

Figure 2.4 Example of poor sample preparation of a sample of PI-stained abalone larvae nuclei with debris and clumping (De Beer, 2003)

Figure 2.5 presents an example of a dual-parameter correlated plot (density plot) which is a two dimensional representation of the distribution of nuclei detected by the forward scatter detector and right angle scatter detector. Each nucleus is represented by a dot in the graph where the intensity of right angle light scatter/side scatter (SSC-Height) is plotted on the vertical axis against the intensity of forward angle light scatter (FSC-Height) on the horizontal axis.

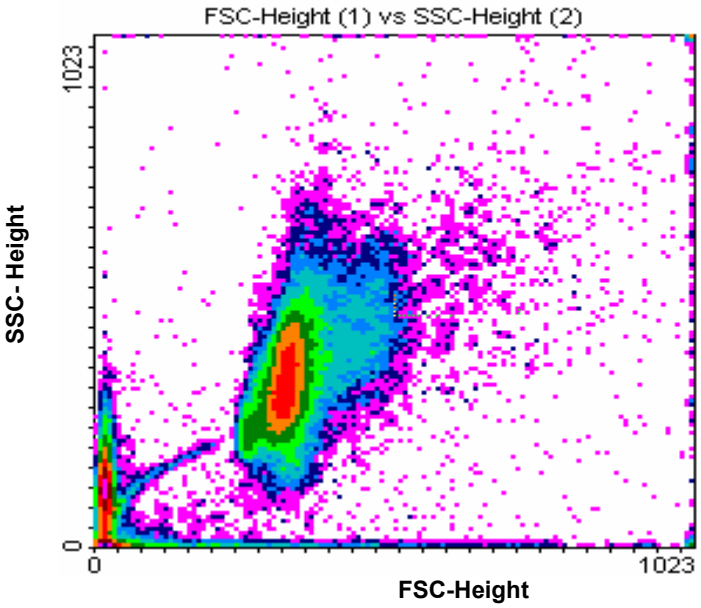

Figure 2.5 A density plot (acquired with WinMDI Version 2.1.3) of PI stained abalone sperm nuclei (nuclear isolation by means of method 2) indicating a population of single nuclei

FSC-Height is indicative of particle size where SSC-Height indicates particle granularity. A population of abalone sperm nuclei is visible as the red-orange area. Debris is visible close to the origin and clumping is visible in blue and purple. The large amount of debris and clumping is an indication of inadequate sample preparation. A further discussion of this sample preparation method continues following Figure 2.8.

The quality of a flow cytometry histogram of nuclear DNA content is indicated by the width of the peak obtained from cells that are in the G_1 phase of the cell cycle. The width is quantified in terms of the coefficient of variation (CV) across the peak. Histograms with CV values of less than 5% are generally considered as of acceptable quality (Dressler, 1990). It is recommended that similar, high concentrations of nuclei is used in order to ensure that good quality histograms with narrow peak widths and low CVs are obtained throughout a series of samples (Ormerod, 2000). In this study, roughly equal amounts of

larvae or sperm were used for preparing each sample, though the concentration of stained nuclei was not determined prior to running of the samples.

Figure 2.6 presents an example of a good quality flow cytometric histogram indicating the number of stained nuclei from abalone sperm detected by a FL2-H fluorescent detector, after preparation by means of method 1 (see section 2.2). The fluorescent density, denoted by a channel number on the horizontal axis, reflects the relative DNA content per nucleus. No debris and only a small amount of aggregates were detected. The peak position i.e. the channel number used to describe the position of the histogram along the horizontal axis, of haploid sperm nuclei is clearly visible at of 51.01.

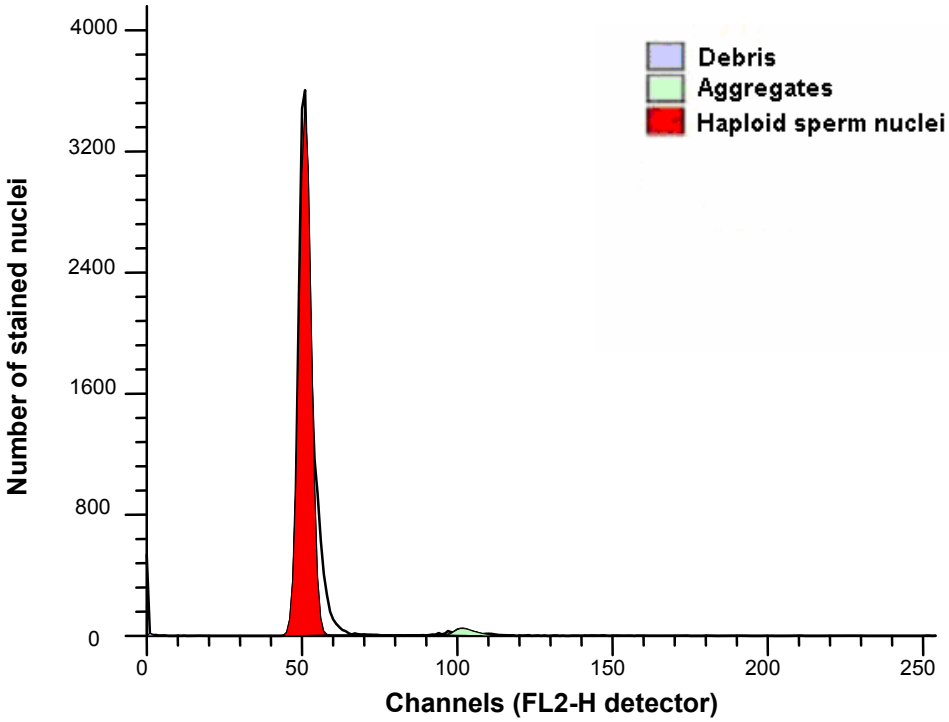

Figure 2.6 A flow cytometric histogram of the number of stained nuclei from abalone sperm detected by the FL2-H fluorescent density detector, after preparation by means of method 1 (see section 2.2)

The methods of nuclear isolation described in section 2.2 gave different quality of results in relation to sperm and larvae. The application of Method 1 to sperm provided histograms of high quality with CV's ranging from 3.24 - 3.69 (Average = 3.47 ± 0.32), and no debris with very little aggregates as indicated by the histogram in Figure 2.6. The application of Method 1 to larvae provided histograms of lower quality, with excessive amounts of debris and aggregates visible in the histogram in Figure 2.7. The G_1 peak

position of diploid larvae nuclei is visible at channel number 112.12 and a portion of diploid nuclei in the S-phase of the cell cycle can also be distinguished (see legend). There is no portion visible as the diploid G_2 phase of the cell cycle in this graph.

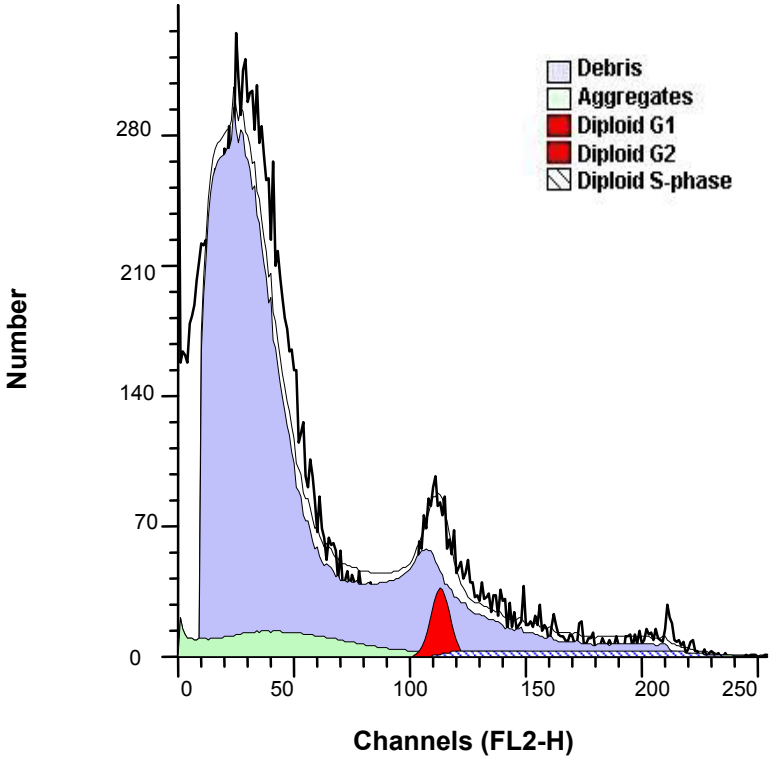

Figure 2.7 A flow cytometric histogram of the number of stained nuclei obtained from diploid abalone larvae 48 hours after fertilization, detected by the FL2-H fluorescent density detector, after preparation by means of method 1 (see section 2.2)

The inferior quality of histograms obtained from the larvae could be due to degradation of larval cells caused by the use of the enzyme trypsin in addition to a detergent (Nonidet-P40). It is expected that the treatment might have been too harsh causing the larval cells to break up into pieces that could not be removed by filtering. This cellular waste probably also absorbed some of the PI fluorescent dye which were detected by the flow cytometer as debris.

The application of Method 2 to sperm provided histograms of lower quality with CV's ranging from 9.48 - 10.73 (Average = 10.11 ± 0.88) which contained excessive amounts of aggregates. This is evident from Figure 2.8 as the green shaded area. The peak position i.e. the channel number used to describe the position of the histogram along the horizontal axis, of haploid sperm nuclei is visible at 24.11.

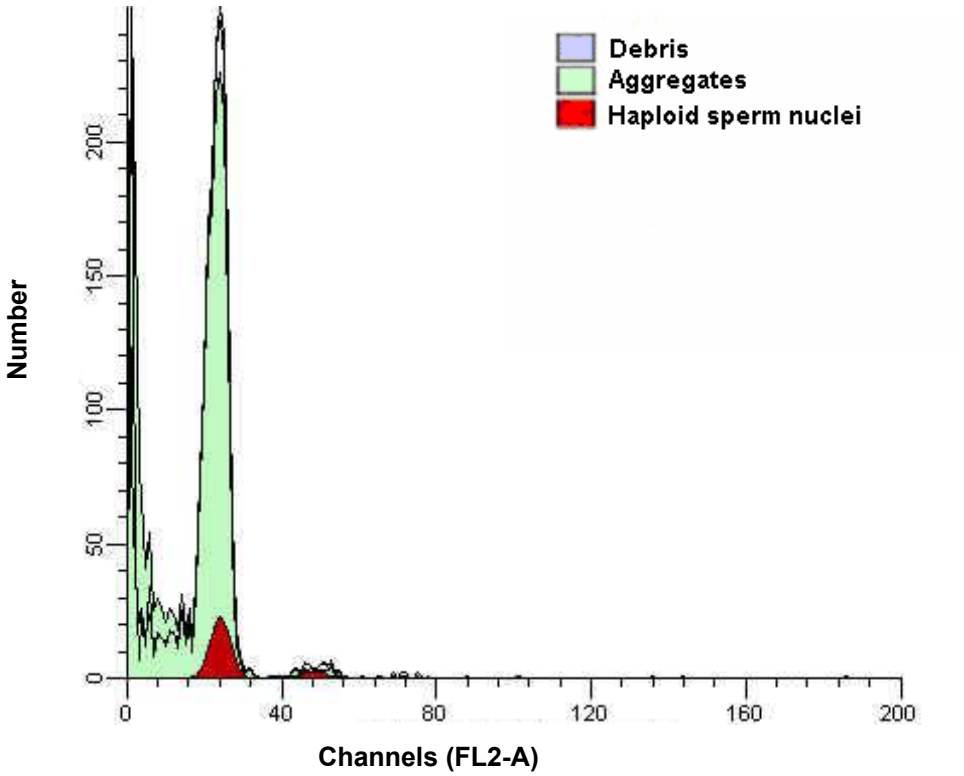

Figure 2.8 A flow cytometric histogram of the number of stained nuclei from abalone sperm detected by the FL2-A fluorescent density detector, after preparation by means of method 2 (see section 2.2)

An explanation for the excessive amounts of aggregates might be the absence of the proteolytic enzyme, trypsin as well as that the detergent Triton X-100 alone was not strong enough to yield singly stained sperm nuclei. In similar studies on mammalian spermatozoa, non-enzymatic methods did not yield quantitative DNA staining because of the highly condensed chromatin in sperm (Otto, Hacker, Zante, Schumann, Göhde and Meistrich, 1979).

Method 3 was successful for isolating and staining nuclei of larval cells, resulting in histograms of high quality (Figures 2.9 - 2.10). The application of Method 3 to diploid larvae provided histograms of high quality with CV's ranging from 2.68 - 5.27 (Average = 3.90 ± 0.84), and little debris and aggregates as indicated by the histogram in Figure 2.9. Diploid nuclei in the S-phase of the cell cycle can also be distinguished clearly (refer to legend).

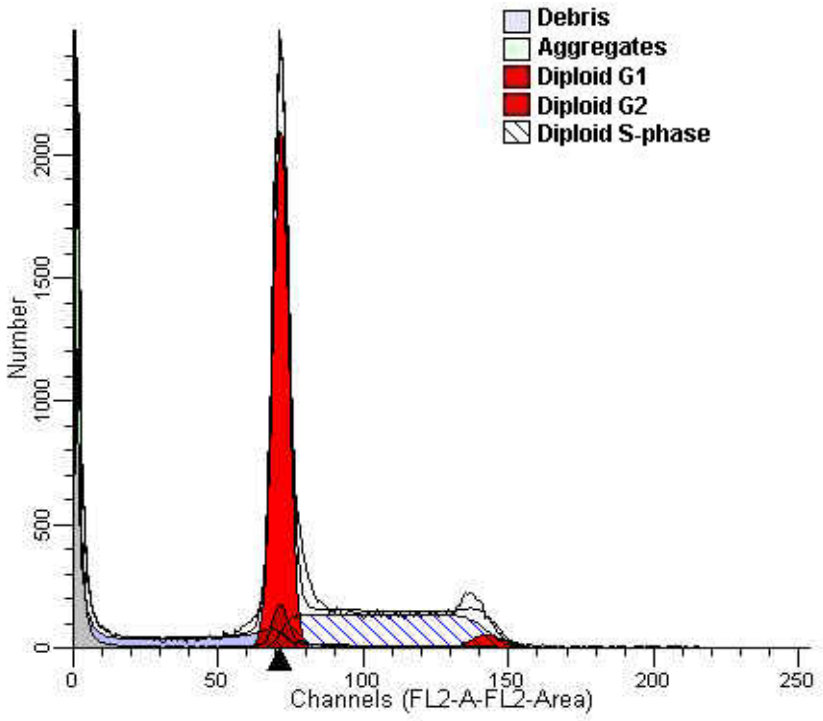

Figure 2.9 A flow cytometric histogram of the number of stained nuclei obtained from diploid abalone larvae 48 hours after fertilization, detected by the FL2-A fluorescent density detector, after preparation by means of method 3 (see section 2.2)

This histogram (Figure 2.9) is a good example of a histogram representative of actively dividing cells, with a lot of cells in the G_1 phase of the cell cycle and fewer cells in the G_2 phase. The G_2 peak (at 142.85) appears characteristically at a channel number double the channel number of the G_1 peak (at 71.42), because nuclei in the G_2 phase contains double the amount of DNA than does G_1 phase nuclei.

In Figure 2.10 the G_1 and G_2 phases of diploid and triploid nuclei are presented. This triploid induction treatment resulted in 94.99% of triploid nuclei, with 5.01% diploid nuclei. The diploid G_2 peak position (at 118.1), which is hidden behind the S-phase of the triploid nuclei, is double that of the G_1 peak position (at 59.05). The triploid G_2 peak position (at 177.49) is also double that of the G_1 peak position (at 88.75). The triploid G_1 peak position is exactly 1.5 times that of the diploid G_1 peak position. This is expected, as amount of dye taken up by the cell is generally proportional to the amount of DNA in the nucleus and triploid nuclei contain 1.5 times the amount of DNA of diploid nuclei (Nell, 2002). Low $CV^{'s}$ of 3.71 for the diploid peak and 3.91 for the triploid peak indicate acceptable quality of the histogram.

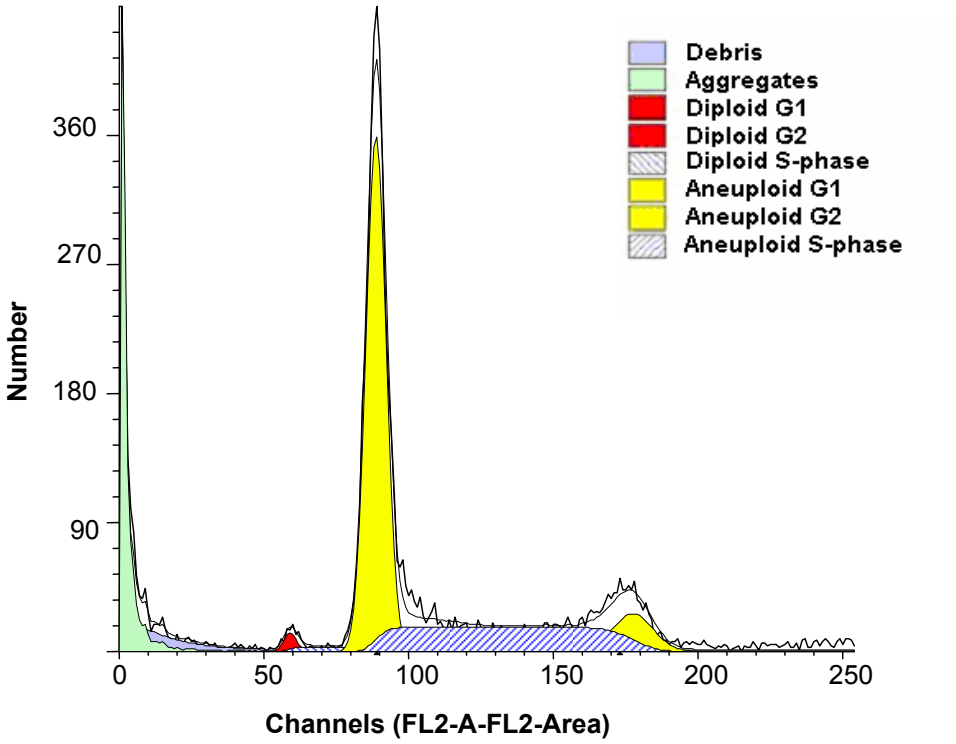

Figure 2.10 A flow cytometric histogram of the number of stained nuclei obtained from induced abalone larvae 48 hours after fertilization, detected by the FL2-A fluorescent density detector, after preparation by means of method 3 (see section 2.2)

Ideally external standards like fluorescent beads should be used to calibrate the flow cytometer on a daily basis. These settings include instrument alignment, setting thresholds for size, light scatter or fluorescence and setting voltages and gains (Dressler, 1990). A DNA reference standard is also essential for the determination of relative DNA content (Dressler, 1990).

The discrepancy in channel number at which the peaks appeared for different samples of the same type (51.01 and 24.11 for sperm in Figures 2.6 and 2.8 and 112.12, 71.42 and 59.05 for G_1 phase of embryos in Figures 2.7, 2.9 and 2.10) may be explained by a variation of dye:DNA ratio because the applied method lacked determination of concentration of stained nuclei, leading to variation in stoichiometric binding of PI to DNA. Failure to set up the instrument according to external standards on a daily basis may also have contributed to such variation in channel numbers. In subsequent samples analyzed by flow cytometry (see Chapter 3) normal diploid larval cells were used as an internal standard. Inconsistent channel numbers amongst samples of the same cell type was

therefore not considered as an insurmountable complication, since for each sample the level of aneuploidy was compared to a normal diploid control which was prepared for flow cytometry together with the expected aneuploid sample.

The aim of this study was to evaluate flow cytometry as a method of identification of triploidy in hydrostatic pressure induced larvae from the South African abalone, *H. midae*. In particular, the method of sample preparation (nuclear isolation) had to be optimised as the interpretability of the flow cytometric histograms is to a great extent dependant on proper sample preparation. The results obtained from this experiment provide convincing evidence that flow cytometry could be considered as a reliable, fast and accurate, though expensive procedure for identification of triploidy in hydrostatic pressure induced larvae from the South African abalone, *H. midae*. The aspiration method (Method 3) where larvae are stained with PI, aspirated, filtered twice and centrifuged was the most successful one for isolating nuclei from abalone larvae, resulting in clearly stained nuclei of relatively high purity and little clumping. This was then the preparation method used to analyze the subsequent samples with.

Alternative methods can be considered for verification of triploidy. The most direct method for detailed ploidy analysis would be to conduct chromosome counts (Beaumont and Fairbrother, 1991; Guo, Cooper, Hershberger *et al*., 1992). This would also give insight into the karyotype of the species, which has not yet been described. Changes of triploid percentages over time are rather a common phenomenon, probably due to differential mortality of triploids and diploids (Liu, Heasman and Simpson, 2004). Verifying triploidy percentage at different developmental stages is thus recommended.

2.4 References

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CHAPTER 3: Induction of triploidy in the South African abalone *H. midae*, using hydrostatic pressure

3.1 Introduction

Cultivation of abalone in South Africa was first attempted in 1981 by the successful spawning of captured specimens to produce spat and juveniles (Sales and Britz, 2001). The South African abalone, *H. midae* was, however, only identified as a suitable marine species for aquaculture in 1990 (Cook and Walmsley, 1990) and from then on steady progress was made towards the successful farming of this species (Henry, 1995). Twelve abalone farms have since been established on the South African coast (Sales and Britz, 2001). Like most other commercially important abalone species, the slow growth rate of *H. midae* is an obstacle in the profitable farming and global competitiveness of this species (Elliott, 2000, Stepto, 1997). Typical growth rates of abalone are two to three centimetres per year. At this rate, two to five years are required for an abalone to reach market size (Hahn, 1989).

The use of genetic manipulations, specifically ploidy manipulation of the maternal genome, to improve growth rate in shellfish has made considerable progress over the past two decades and is considered the most comprehensive area of abalone genetics examined thus far (Beaumont and Fairbrother, 1991; Elliott, 2000). Triploidy induction in molluscs is a technology that is increasingly used by many commercial aquaculture farms worldwide to improve production. Triploidy is induced in fertilized eggs during Meiosis I or Meiosis II by suppressing formation of either the first or the second polar body (Boudry, Barré and Gérard, 1998). The result is that each cell nucleus contains one additional set of chromosomes (contributed by the polar body) (Gérard, Ledu, Phélipot and Naciri-Graven, 1999). The importance of triploidy lies in the presumption that homologous chromosomes in the germ cells of adult triploids cannot synapse at meiosis. Triploid adult animals are thus inclined to be functionally sterile (Beaumont and Fairbrother, 1991; Kapuscinski, 2000).

Sterility is desirable because of the following reasons: Energy usually diverted to gamete production becomes available for somatic growth; therefore adult triploids should grow faster (Beaumont and Fairbrother, 1991; Elliott, 2000). Yang, Chen and Ting (1998) also noted that because of larger nuclei in triploid cells, they can accommodate increased DNA and this leads to concomitant increase in overall cell size. The ripe gonads of adult

animals in breeding season often render these animals unmarketable, while depleted glycogen levels during this period also negatively affects flavour and quality (Beaumont and Fairbrother, 1991; Boudry *et al*., 1998; Chao, Yang, Tsai, Liang and Liao, 1993). This problem can be overcome by producing sterile animals. Also, the risk of accidental introduction of non-native species from aquaculture facilities into the environment is decreased as such animals will not be able to reproduce (Beaumont and Fairbrother, 1991; Kapuscinski, 2000). On a genetic level, triploidy potentially induces higher mean heterozygosity because of the higher probability that triploids possess two or three different alleles per locus (Boudry *et al*., 1998; Guo, Cooper, Hershberger *et al*., 1992). This results in the phenomenon of "heterosis" or hybrid vigour which may be manifested as faster growth, higher viability or increased fitness (Beaumont and Fairbrother, 1991) as well as positively influencing feeding rate, absorption rate and growth efficiency (Garnier-Géré, Naciri-Graven, Bougrier *et al*., 2002).

Triploidy is accomplished by induction techniques, including chemical induction (Cytochalasin B or 6-DMAP), thermal shock (either heat or cold shock), and hydrostatic pressure shock or by crossing tetraploids and diploids (Beaumont and Fairbrother, 1991; Allen and Guo, 1996). Triploidy induction by inhibition of both the first and second polar bodies has been attempted in the Pacific abalone, *H. discus hannai* (Arai, Naito and Fujino, 1986; Wang, Zhang, Wang *et al*., 1990; Zhang, Wang, Chang *et al*., 1998), Small abalone, *H. diversicolor* (Yang, Chen and Ting, 1998; Yang, Ting and Chen, 1998), South African abalone, *H. midae* (Stepto and Cook, 1998), Pacific red abalone, *H. rufescens* (Maldonado *et al*., 2001), Tropical abalone, *H. asinina* (Norris and Preston, 2003) and Australian blacklip abalone, *H. rubra* (Liu, Heasman and Simpson, 2004), with hydrostatic pressure, thermal, CB or 6-DMAP treatment. However, few of the studies provided techniques that combined high triploid percentages with reasonable larval survival (Liu *et al.,* 2004). An in depth evaluation of relative growth and reproduction performances of triploid and diploid siblings is also lacking (Zhang *et al*., 1998) and triploid lines will need to be produced on a commercial scale to evaluate survival and normality of triploid larvae, growth characteristics, maturity and market acceptance (Elliott, 2000). According to Liu, Heasman and Simpson (2004), the question whether abalone farming industries can exploit the advantages conferred by triploidy, namely sterility and faster growth, has not yet been accurately assessed. What is needed is the further validation of induction techniques and transfer of the technology from other commercial species. The production

of tetraploids for future triploid production is also a field that will need to be further studied in abalone (Elliott, 2000).

The aim of this project is 1) to determine whether triploidy can be successfully induced in the South African abalone, *H. midae*, through the inhibition of the extrusion of the second polar body by using hydrostatic pressure shock and 2) to validate flow cytometry as a method for the identification and verification of triploidy in *H. midae*. The method of hydrostatic pressure shock has not been experimented with on the South African abalone. Hydrostatic pressure shock is a physical method of induction and has the advantages of being cost effective and non-toxic. Hydrostatic pressure shock has been applied with success in other abalone species (Arai, Naito and Fujino, 1986) on which basis it was chosen as a relevant induction method. During chromosome set manipulation, the determination of ploidy is a critical and necessary procedure (Yang, Gallivan, Guo, *et al*., 2000). After reviewing several methods for assessing the success of triploidy induction, flow cytometry appeared to be the most appropriate method. Flow cytometry is a rapid, powerful method for measuring DNA that has been used with success in determination of ploidy in shellfish since 1983 (Allen, 1983). It was considered to be a reliable method for the determination of ploidy in the induced larvae.

3.2 Experimental design

The parameters used in the experiment to determine the optimum conditions for induction of triploidy were hydrostatic pressure levels and duration of pressure treatment. A series of different combinations of these two treatment parameters were applied to fertilized abalone eggs. The treatments were applied 24 minutes after fertilization, in order to prevent the extrusion of the second polar body (PB2), which is released 33 - 36 minutes after fertilization in *H. midae* (Stepto, 1997; Stepto and Cook, 1998).

Two consecutive experiments were conducted. **Experiment A** was conducted to establish the broad range of pressure and time treatments that will affect induction of triploidy in *H. midae*. A split-plot experimental design (Table 3.1) was used, which included combinations of five different pressure treatments ranging from 20 to 50 MPa and four different time treatments ranging from 0 to 10 minutes. Untreated control groups were maintained for each treatment.

Table 3.1 A summary of the different combinations of pressure and time treatments conducted on abalone larvae in Experiment A, according to a split-plot experimental design

Duration of treatment/time	Pressure Treatment				
	20 MPa	30 MPa	35 MPa	40 MPa	50 MPa
0 min	Control A	Control B	Control C	Control D	Control E
5 min	Treatment A1	Treatment B1	Treatment C1	Treatment D1	Treatment E1
7 min	Treatment A2	Treatment B2	Treatment C2	Treatment D2	Treatment E2
10 min	Treatment A3	Treatment B3	Treatment C3	Treatment D3	Treatment E3

Experiment B was developed by the selection of an additional range of pressure and time treatment combinations based on preliminary results of levels of triploidy obtained in larval samples from Experiment A. The objective with Experiment B was to identify a more refined range of pressure and time treatments that will allow further optimization of the induction protocol for triploidy in *H. midae*. A rotatable experimental design (Table 3.2) was used to create various combinations of five different pressure treatments ranging from 0 to 30 MPa and three different time treatments ranging from 5 to 15 minutes, some of which overlapped with treatments of Experiment A. Untreated control

groups were maintained for each treatment. Experiment B was also repeated for a second time.

Table 3.2 A summary of the different combinations of pressure and time treatments conducted on abalone larvae in Experiment B, according to a rotatable experimental design

Duration of treatment/time	Pressure Treatment				
	0 MPa	15 MPa	20 MPa	25 MPa	30 MPa
5 min	Control F	Treatment F1	Treatment F2	Treatment F3	Treatment F4
10 min	Control G	Treatment G1	Treatment G2	Treatment G3	Treatment G4
15 min	Control H	Treatment H1	Treatment H2	Treatment H3	Treatment H4

3.3 Materials and Methods

3.3.1 Pressure Inductions

Spawning and fertilization

The material used for the pressure inductions consisted of freshly spawned eggs and sperm obtained from broodstock at the Irvin & Johnson Abalone Culture Division situated at Danger Point on the Cape south western coast. An average of three females and three males were used per spawning. Males and females were kept and spawned in separate basins. Spawning was induced with the Hydrogen Peroxide technique as specified by Hahn (1989) and refined by Irvin & Johnson (Figures 3.2 and 3.3). The spawned gametes were kept in filtered seawater at a temperature of 17˚C until fertilization. All fertilizations were carried out within two hours of spawning at the standard temperature of 17˚C to ensure gametes of good quality (Stephano, 1992). Shortly before fertilization, eggs were siphoned from the bottom of the spawning basin into a clean glass beaker and filtered through 400 – 500 µm mesh sieve to exclude debris, faeces and mucus. A monolayer of eggs was collected onto a 100 µm mesh sieve partially immersed in filtered, UV-sterilized seawater. Sperm was collected from the top layer of water in the spawning basin and added in excess to the eggs with the time being recorded. A time of ten minutes was allowed for fertilization after which fertilized eggs were gently rinsed with clean seawater for seven minutes. The fertilized eggs were collected into a glass beaker in a concentrated form and transferred to the pressure apparatus for the pressure treatments.

Induction

The initial pressure inductions were conducted with a high volume apparatus developed by AquaEco for the use in grass carp. These inductions resulted in early embryos containing bubbles of gas in the perivitelline space, which caused early mortality (Figure 3.4). It was suspected that these gas bubbles formed because of the high volume of the cylinder (1.2 litres) and the subsequent failure to concentrate the eggs sufficiently. A new pressure apparatus, with a reduced volume, was therefore developed for specialized application in abalone. It consisted of a stainless steel cylinder with a maximum volume of 150 ml, a copper headpiece fitted with rubber o-rings to fit tightly onto the cylinder, a pressure gauge, a hydraulic jack and a stainless steel frame (Figure 3.6). Three interchangeable stainless steel discs can be inserted to decrease the

volume by 20 ml respectively. This pressure apparatus was used for all the subsequent inductions.

The fertilized eggs were gently poured into the cylinder (volume 150 ml) and air bubbles were removed by gently tapping the side of the cylinder with a nylon hammer. The headpiece was adjusted and the cylinder with headpiece placed on the stainless steel frame. At 24 minutes after commencement of fertilization (Stepto, 1997), pressure was applied to the fertilized eggs by manipulation of the hydraulic jack. The applicable pressure was applied for the required time (see Tables 3.1 and 3.2) after which the pressure was released instantly by depressurizing the hydraulic jack. The fertilized, shock induced eggs were transferred to an 11 litre plastic basin containing stagnant, filtered seawater at 17˚C where they were kept until hatching. Throughout, care was taken to handle the eggs as gently as possible. This procedure was repeated for every treatment.

For each treatment a portion of fertilized eggs was sampled as a control group before pressure treatment was conducted. This control portion consisted of a similar quantity (150 ml) of fertilized eggs from the same batch as those used for pressure inductions. The control was transferred to the plastic basin before its equivalent treatment group was subjected to pressure. Consequently, the control consisted of eggs and sperm from the same parents as the eggs and sperm used for the groups receiving pressure treatment. The control received no treatment (pressure = zero; time = zero) but were directly transferred to an 11 litre plastic basin containing stagnant, filtered seawater at 17˚C where they were kept until hatching.

3.3.2 Estimation of survival

Survival of larvae from the treated and the control groups were estimated approximately 20 hours post fertilization. From the basin with larvae, a small area was gently stirred with a sterile plastic pipette to get an even suspension of live and dead larvae lying on the bottom. Because of concern for adequate larval numbers needed for later stages of experimentation, care was taken not to disturb all the larvae in the basin. A 2 ml random sample was quickly taken from the larvae in suspension and placed in 3 ml seawater in a 60 mm plastic Petri dish with a rough grid scratched on the bottom. The sample was examined under four times magnification on a Nikon stereomicroscope. Numbers of normal, abnormal and dead larvae were counted and percentage normal and living larvae were calculated for each sample. Eventually the percentage of morphologically normal

larvae was used as a measure of larval survival and used for further calculations (Yang, Chen and Ting, 1998; Yang, Ting and Chen, 1998). Subsequently each group of larvae was transferred to a 100 litre hatchery bin, with air supply and a constant flow of fresh, filtered seawater. Cleanliness was maintained throughout these procedures to avoid possible contamination.

3.3.3 Sample collection and preservation

The procedure for sample collection and preservation is very important since it influences success of the flow cytometric analysis to follow. Several methods were tested on normal, untreated larvae before one was eventually chosen as the preferred method of collection to be used during experimentation. All collection procedures were carried out in a clean, sterile environment as far as possible. Surfaces and hands were cleaned with alcohol and all equipment alcohol sterilized and rinsed with filtered seawater between samples. Three cryovials (volume 1.8 ml) were collected for each method (Figure 3.7). The different methods for collection and preservation of larvae that were compared are explained below:

Method 1

Larvae of approximately one hour old were concentrated onto a 100 µm nytex screen and directly scooped into sterile 1.8 ml plastic cryovials. Ice cold Vindeløv buffer (1.5 ml) (Vindeløv and Christensen, 1994; see Appendix 2) was added and larvae were transported on dry ice to the laboratory where DMSO (150 µl) was added. Samples were frozen at -20˚C (Stepto, 2003).

Method 2

One hour old larvae were collected in seawater into a sterile 50 ml plastic falcon tube and transported to the laboratory on ice where they were concentrated onto a 100 µm nytex screen and scooped into sterile 1.8 ml plastic cryovials. Ice cold Vindeløv buffer (1.5 ml) and DMSO (150 µl) were added immediately and the samples were frozen at -20˚C (Stepto, 2003).

Method 3

One hour old larvae were collected in seawater into a sterile 15 ml plastic falcon tube and transported to the laboratory on ice. After centrifuging at 800 rpm for 10 minutes, the supernatant was discarded and the larvae resuspended in 1.5 ml ice cold Vindeløv buffer

with 150 µl DMSO. The suspension was transferred to sterile 1.8 ml plastic cryovials and frozen at -20˚C (Stepto, 2003).

Method 4

Samples of 500 µl were taken with a sterile plastic pipette into sterile 1.8 ml plastic cryovials, from a bin containing larvae of approximately one hour old. Ice cold Vindeløv buffer (1 ml) was added and the samples transported to the laboratory on ice where, DMSO (100 µl) was added before the samples were frozen at -20˚C (Stepto, 2003).

Method 5

One hour old larvae were collected in seawater into a sterile 50 ml plastic falcon tube and transported to the laboratory on ice. Subsequently samples of 500 µl were taken into sterile 1.8 ml plastic cryovials, using a sterile plastic pipette. Ice cold Vindeløv buffer (1 ml) and DMSO (100 µl) were added before the samples were frozen at -20˚C.

Method 6

Larvae of approximately 44 hours old were concentrated onto a 100 µm nytex screen and rinsed into a sterile 50 ml falcon tube with 10 ml 0.075 M KCl. Pre-treatment with KCl has been indicated in aiding nuclear isolation for subsequent flow cytometry (Yang *et al*., 2000). The larvae were left suspended in the KCl for ten minutes and then concentrated onto a 100 µm nytex screen and scooped into sterile 1.8 ml plastic cryovials. A sterile plastic pipette was used to add 1.5 ml of 75 percent ethanol to the larvae. The samples were left at room temperature until analysis (Yang *et al*., 2000).

Sperm samples were also collected to investigate its use as an internal standard for flow cytometry. Five cryovials were collected for each of the two methods explained below.

Method 7

Sperm, suspended in seawater, were collected into sterile 15 ml plastic falcon tubes and transported to the laboratory on ice. After centrifuging at 800 rpm for 10 minutes, the supernatant was discarded and the sperm resuspended in 1.5 ml ice cold Vindeløv buffer with 150 µl DMSO. For each falcon tube the suspension was transferred to a sterile 1.8 ml plastic cryovial and frozen at -20˚C (Stepto, 2003).

Method 8

A sterile plastic pipette was used to transfer 500 µl sperm, suspended in seawater, into sterile 1.8 ml plastic cryovials. Vindeløv buffer (1 ml) was added and the samples were

transported to the laboratory on ice. DMSO (150 µl) was added before the samples were frozen at -20˚C (Stepto, 2003).

Preferred method

The preferred method for the collection and preservation of larval samples that was applied through the remainder of the study is a combination of methods 1 and 6 as described above. This method is preferred because it rendered the most appropriate samples with well isolated nuclei for analysis through flow cytometry. Samples also stayed well preserved until used for flow cytometry (2 - 3 weeks in this case). Larvae of approximately 44 hours old were concentrated onto a 100 µm nytex screen and rinsed into a sterile 50 ml plastic falcon tube with 10 ml 0.075 M KCl. The larvae were left suspended in the KCl for ten minutes and then again concentrated onto a 100 µm nytex screen and scooped into sterile 1.8 ml plastic cryovials. Ice cold Vindeløv buffer (1.5 ml) and DMSO (150 µl) was added before the samples were frozen at -20˚C.

3.3.4 Flow Cytometry

Sample preparation (nuclear isolation and staining)

The aim of sample preparation is to obtain single nuclei, with minimum degradation of the DNA and minimum debris and clumping/aggregation. Proper sample preparation and staining procedures are essential to obtain high quality flow cytometry histograms (Ormerod, 1999). Different methods of sample preparation were evaluated, as described in Chapter 2, until acceptable flow cytometry histograms were obtained.

The following standardized procedure of sample preparation was followed:

Samples were taken from the -20˚C freezer and thawed at room temperature. From each sample, 50 µl of concentrated larvae was taken and 50 µl MPBS (Allen, 1983; see Appendix 2), and 500 µl Propidium iodide (PI) staining solution (Norris and Preston, 2003; see Appendix 2), was added to it. The suspension was then aspirated through a 1 ml syringe fitted with a 26½ G needle (Allen *et al*., 1993; Ormerod, 1999) until no more clogging of the needle's tip occurred. The cell suspension was now filtered through a 25 µm nytex screen twice (Allen, 1983; Ormerod, 1999) and centrifuged for 5 minutes at 5000 rpm. The supernatant was removed and the pellet of stained larval nuclei resuspended with a micropipette in 500 µl MPBS in a plastic eppendorf tube. The tube was wrapped in foil and kept on ice to ensure stability of the PI (Sigma Product

Information, 2003) and samples were analyzed within 5 hours from staining (Chrissman and Hirons, 1994).

Fluorescence microscopy

Before running the samples on the flow cytometer, they were first examined on a Nikon Eclipse E400 microscope using fluorescence microscopy. This was done to affirm that the sample is prepared satisfactorily for flow cytometry, without excess debris or an excessive number of cell clumps/aggregates (Ormerod, 1999). Samples where separate, brightly stained, red nuclei were visible were approved to be analysed by flow cytometry (Chapter 2: Figure 2.3).

Flow cytometry

Flow cytometry records the fluorescence intensity of stained nuclei, which is digitally converted into quantitative information (Thorpe and Thorpe, 2000). The curvature graphs obtained via flow cytometry describes the distribution of fluorescence signals from nuclei by a frequency distribution histogram. The peak position of the histogram is measured by channel numbers on the horizontal axis and reflects the relative DNA content per nucleus. The number of nuclei recorded is shown on the vertical axis (Yang *et al*., 2000). The area under the curve represents the relative contribution of a certain ploidy (Allen, 1983) (Chapter 2: Figures 2.6 – 2.10).

The PI stained samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer with a He/Ne laser, with wavelength 488 nm (Figure 3.8). Prior to running, samples were vortexed to get the nuclei in suspension and transferred to a 5 ml flow cytometry tube. The instrument settings were adjusted appropriately via the Apple Mackintosh computer connected to the flow cytometer. For each sample, a minimum of 20 000 nuclei were acquired at a flow rate of 100 to 250 nuclei per second to generate histograms.

For each group of treatments, a diploid control group was used as internal standard and its nuclear DNA content compared with that of the treated groups. Flow cytometry histograms were plotted using the curve-fitting program ModFit (Verity Software House, Topsham, ME, USA) and statistical analysis was done on the resulting percentages of ploidy.

Figure 3.1 Layout of a commercial abalone farm at Gansbaai, South Africa

Figure 3.2 Spawning of male abalone (Sorgeloos *et al*., 1997)

Figure 3.3 Spawning of female abalone (Sorgeloos *et al.*, 1997)

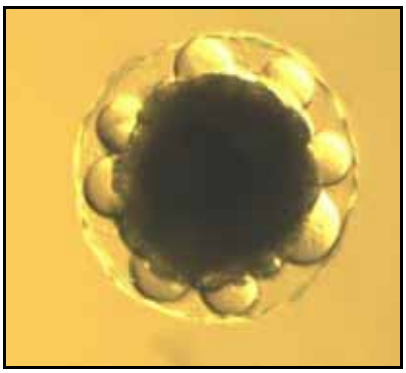

Figure 3.4 Fertilized egg with bubbles in perivitelline space (De Beer, 2003)

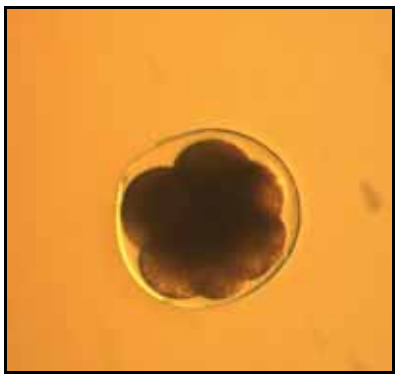

Figure 3.5 Normal fertilized abalone egg (4 hours) (De Beer, 2003)

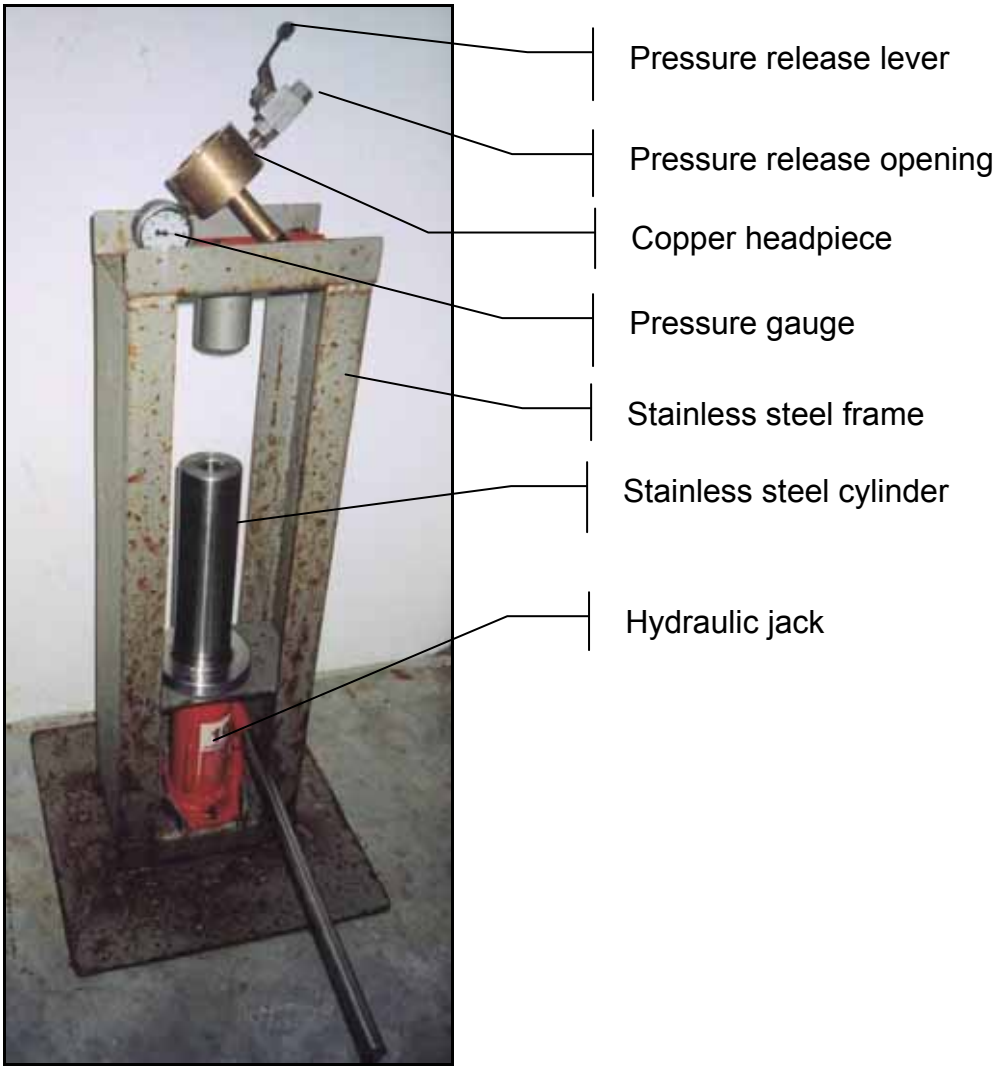

Figure 3.6 Pressure apparatus built for triploidy induction by hydrostatic pressure in abalone

Figure 3.7 Example of sterile plastic cryovials (Corning®) used for larval sample collection

Figure 3.8 Becton Dickinson FACSCalibur flow cytometer at Tygerberg campus, Department Anatomy and Histology, University of Stellenbosch used for analysis of samples

3.4 Results

The data were analyzed using SAS (Statistical Analysis Systems) and Microsoft Excel. Data for survival and triploidy are presented separately. Analyses of variance (ANOVA) were conducted and the linear regression model was fitted for the data. Survival was determined at 20 hours after fertilization, during the trochophore stage of larval development. Triploidy was determined on the basis of preserved samples taken at 48 hours after fertilization during the veliger stage of larval development.

A sample from the control group was taken for each treatment. These controls consisted of a random mixture of fertilized eggs, from the same spawning as the treated eggs, which received no treatment (pressure = zero; time = zero).

3.4.1 Levels of Triploidy

The ANOVA and the Linear Regression according to the General Linear Model Procedure were conducted with the use of SAS (SAS Institute Inc., 1996). Graphs were constructed with Microsoft Excel (Microsoft ® Excel 2002) and GraphPad Prism (Prism 4 for Windows, Version 4.00, April 3 2003).

3.4.1.1 Analysis of Variance of the level of triploidy on the basis of different treatments

The control samples yielded a zero percentage of triploidy over all treatment groups as expected. A log transformation was done on the data for percentage of triploidy in the treated groups in order to avoid a false assumption of normal distribution. An overall mean of 89.788 ± 3.428 was obtained in terms of percentage of triploidy over all treatments together with a coefficient of variance (CV) of 4.825 percent. The low standard deviation and CV gave an indication of limited variation within the treated groups in terms of percentage of triploidy.

The results of an ANOVA for percentage of triploidy on the basis of different treatments are presented in Table 3.3. The null hypothesis (H_o) of no difference in the level of triploidy on the basis of the different treatments is accepted on the basis of a P-value of 0.654 (P>0.05). This implies that the observed differences in the levels of triploidy obtained from the different treatments were not statistically significant between treatments. Separate *t*-tests (LSD) also indicate that the level of triploidy of treatment groups does not vary significantly with different pressures or with different times.

Table 3.3 Results of an Analysis of Variance of the percentage of triploidy in abalone obtained from different treatments, i.e. pressure and duration

Source	df	Sum of squares	Mean square	F	P
Treatment	24	0.957	0.040	0.84	0.6536
Error	12	0.567	0.047		
Corrected total	36	1.524			

None of the time or pressure treatments can therefore be distinguished, on the basis of these results, as not suitable for the induction of triploidy in the abalone, *H. midae*.

3.4.1.2 Regression analysis of relationship between levels of triploidy and pressure as variable at fixed durations of treatment

A linear regression between percentage triploidy and pressure treatments was fitted for each time period/duration of treatment as presented in Table 3.4 and Figure 3.9. The R^2 value of 0.963 indicates that the linear regression model presents a proper fit to the data.

Table 3.4 Results of Linear Regression between percentage triploidy and pressure treatments at fixed durations of treatment, with estimates of intercepts and slopes

Parameter		Estimate	Standard Error	T Value	P- value
Intercept	5 min	45.065	16.500	2.73	0.0105
	7 min	83.174	32.506	2.56	0.0158
	10 min	89.535	16.500	5.43	<.0001
	15 min	55.135	30.245	1.82	0.0783
Slope	5 min	1.328	0.569	2.33	0.0266
	7 min	0.290	0.893	0.32	0.7476
	10 min	0.281	0.569	0.49	0.6257
	15 min	1.492	1.281	1.16	0.2535

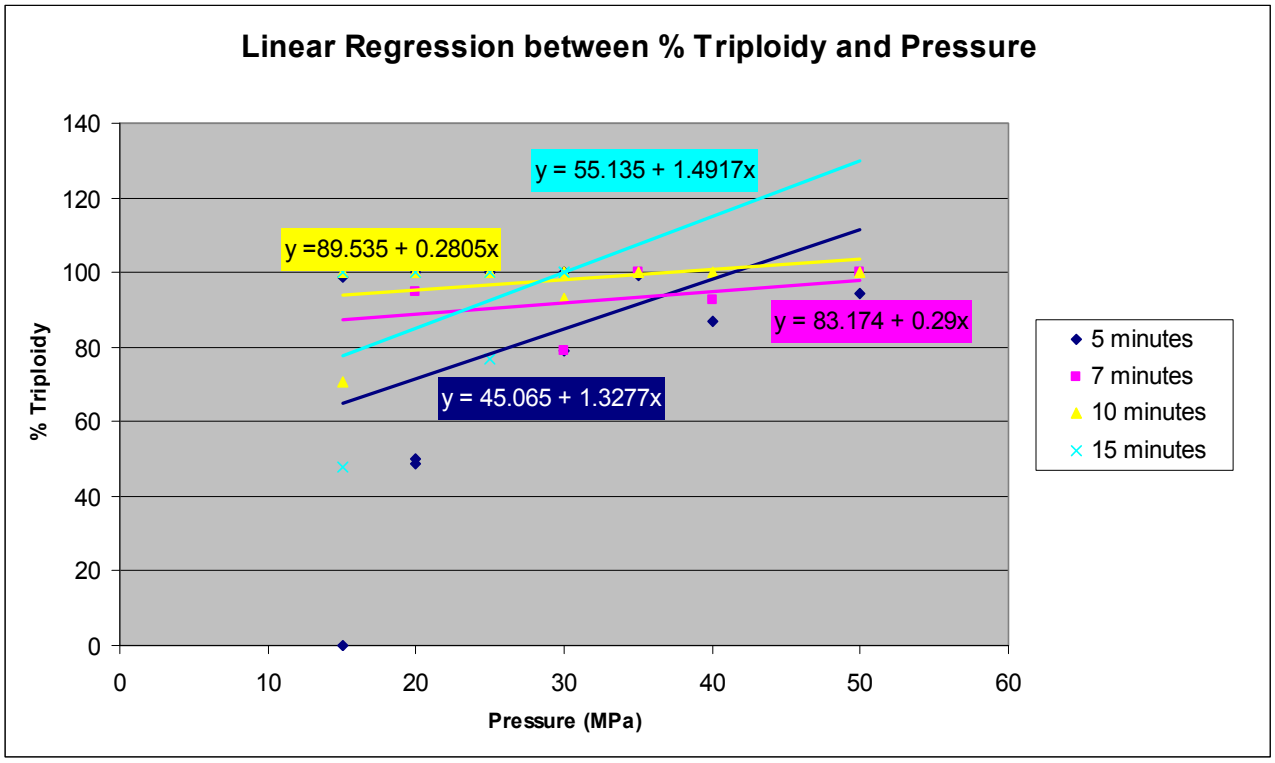

Figure 3.9 Linear regressions between percentage triploidy and pressure treatment for different time periods/durations

5 minutes: At time 5 minutes, the linear regression between percentage triploidy and pressure treatment indicates a steep slope with a gradient of 1.328. This, along with the standard error of the slope, indicates that attainment of triploidy in treated larvae is variable across different pressure intensities at time 5 minutes. The slope may range from 0.759 to 1.897 (SE=0.569). The P-value of 0.0266 ($P<0.05$) results in rejection of the null hypothesis that the slope does not differ from 0, i.e. different pressure treatments have a significant influence on the percentage of triploidy at a treatment regime of 5 minutes.

7 minutes: At time 7 minutes the linear regression between percentage triploidy and pressure treatment indicates a more gradual slope with a gradient of 0.290 when compared to the linear regression at time 5 minutes. This, along with the standard error of the slope, indicates that attainment of triploidy in treated larvae is less variable across different pressure intensities at time 7 minutes than at time 5 minutes. The slope may range from -0.603 to 1.183 (SE=0.893). The P-value of 0.7476 ($P>0.05$) results in acceptance of the null hypothesis that the slope does not differ from zero, i.e. different

pressure treatments do not have a significant influence on the percentage of triploidy at a treatment regime of 7 minutes

10 minutes: At time 10 minutes the linear regression between percentage triploidy and pressure treatment indicates a similar trend as at time 7 minutes. The gradual slope with a gradient of 0.281 together with the standard error of the slope indicates that attainment of triploidy in treated larvae is less variable across different pressure intensities at time 10 minutes than at time 5 minutes. The slope may range from -0.289 to 0.850 (SE=0.569). The P-value of 0.6257 (P>0.05) results in acceptance of the null hypothesis that the slope does not differ from zero, i.e. different pressure treatments do not have a significant influence on the percentage of triploidy at a treatment regime of 10 minutes.

15 minutes: At time 15 minutes the linear regression between percentage triploidy and pressure treatment indicates a similar trend as at time 5 minutes. The steep slope with a gradient of 1.492 indicates that attainment of triploidy in treated larvae is variable across different pressure intensities at time 15 minutes. The slope may range from 0.210 to 2.773 (SE=1.281). Despite the trend observed in the graph, the P-value of 0.2535 (P>0.05) results in acceptance of the null hypothesis that the slope does not differ from zero, i.e. different pressure treatments do not have a significant influence on the percentage of triploidy at a treatment regime of 15 minutes.

Grouping of common slopes

The hypothesis of equal slopes for the lines 7 minutes and 10 minutes (7+10) was tested by carrying out a *t*-test. The data were found to be in agreement with the hypothesis of a common slope on which basis the data were considered for combined analysis. This was done in order to reduce the error and thus increase overall precision of predicting optimal time-pressure treatment regimes. Likewise, for 5 minutes and 15 minutes (5+15), the data were found to be in agreement with the hypothesis of a common slope. In each case *t*-tests were also carried out to test the hypothesis of equal intercepts, for the lines (7+10) minutes and the lines (5+15) minutes respectively. The estimates for the common slopes and intercepts of these lines and their 95 percent confidence intervals are displayed in Tables 3.5 and 3.6.

Table 3.5 Results of Linear Regression between percentage triploidy and pressure treatments, on the basis of a combined analysis, with estimates of common slopes and intercepts

Parameter		**Estimate**	**Standard error**	**T-value**	**P-value**
Intercept	(5+15) min	53.854	13.420	4.01	0.0003
	(7+10) min	90.325	13.803	6.54	<0.0001
Slope	(5+15) min	1.174	0.493	2.38	0.0229
	(7+10) min	0.197	0.442	0.45	0.6590

Table 3.6 95 Percent confidence intervals for common slopes (combined lines)

95% Confidence intervals		**5+15 min**	**7+10 min**
	Lower	0.139	-0.740
	Upper	2.210	1.134

Table 3.7 95 Percent confidence intervals for individual slopes (separate lines)

95% Confidence intervals		**5 min**	**7 min**	**10 min**	**15 min**
	Lower	0.075	-2.552	-0.972	-1.802
	Upper	2.581	3.132	1.533	4.786

Tables 3.6 and 3.7 display the lower and upper limits for the 95 percent confidence intervals for slopes of the lines. Table 3.6 presents the results of the combined analysis of treatments (7+10) minutes as well as (5+15) minutes. The 95 percent confidence interval for slope at time (7+10) minutes includes the value of zero, therefore, it can be assumed that it is possible to attain a line with gradient zero (no variation across different pressures) for the 7 and 10 minute treatments. The 95 percent confidence interval for slope at time (5+15) minutes does not include the value of zero, indicating that a linear regression with 95 percent confidence for line (5+15) minutes show more variation in percentage triploidy at treatment durations of 5 minutes or 15 minutes (with an increase in percentage triploidy with an increase in pressure).

The graph (Figure 3.10) obtained by means of GraphPad Prism (version 4.00, April 2003) presents the linear regression between percentage triploidy and pressure treatment on

the basis of combined analysis with 95 percent confidence intervals for the common slopes of the combined treatments, i.e. (7+10) and (5+15) minutes.

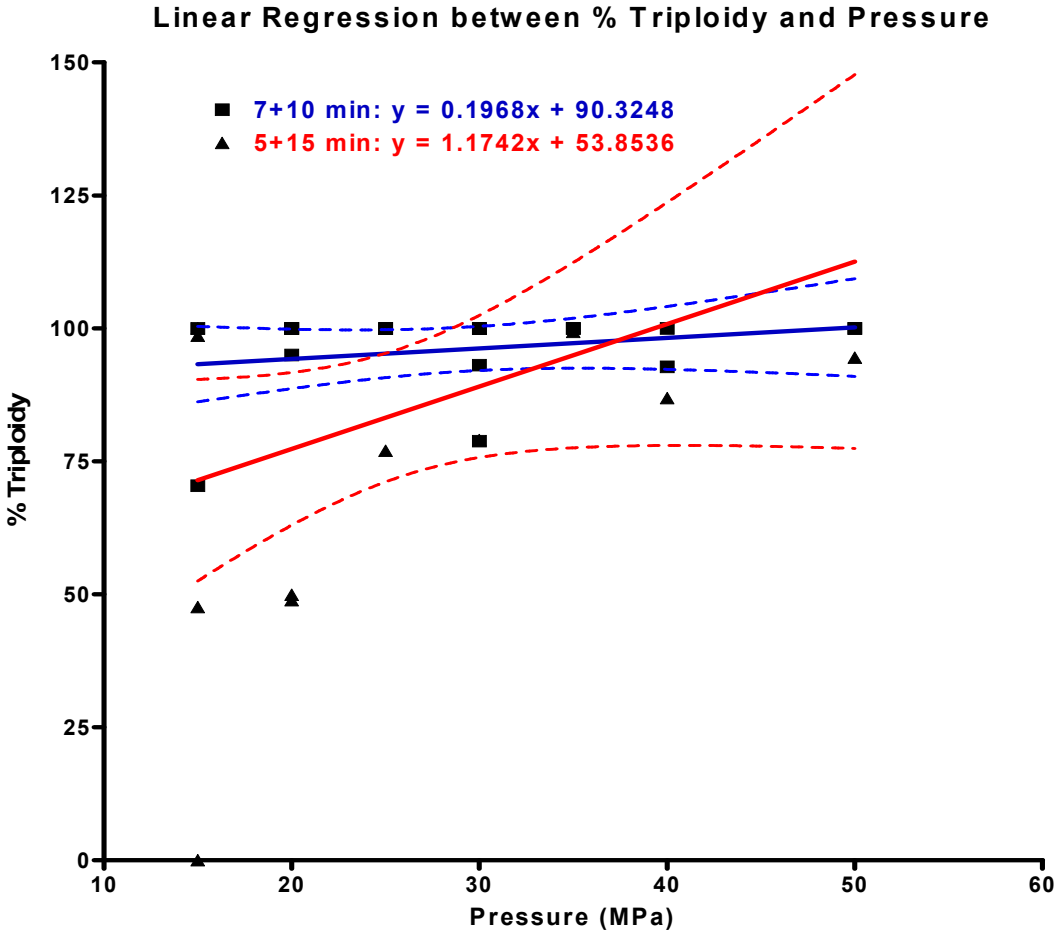

Figure 3.10 Linear regression between percentage triploidy and pressure treatment on the basis of combined analysis of times (7+10) and (5+15) minutes, with 95 percent confidence intervals of slopes

The line (7+10) minutes have a much narrower confidence interval than the line (5+15) minutes. This reflects less uncertainty about the percentage triploidy for each pressure treatment when treatment duration is 7 or 10 minutes than when treatment duration is 5 or 15 minutes. When looking at the lower confidence interval bands, the lowest point for the line (7+10) minutes lies at 86.272, while for the line (5+15) minutes, the lowest point on the lower band is 52.253. According to this graph, percentage triploidy attained when treating fertilized eggs for 7 or 10 minutes will consequently be at least 86.272 percent regardless of the pressure (within the tested range) that is used.

3.4.1.3 Regression analysis of relationship between levels of triploidy and duration/time as variable at fixed pressures

The linear regression between percentage triploidy and duration of treatment (time) was fitted for each pressure. The R^2 value of 0.967 indicates that the linear regression model presents a proper fit to the data.

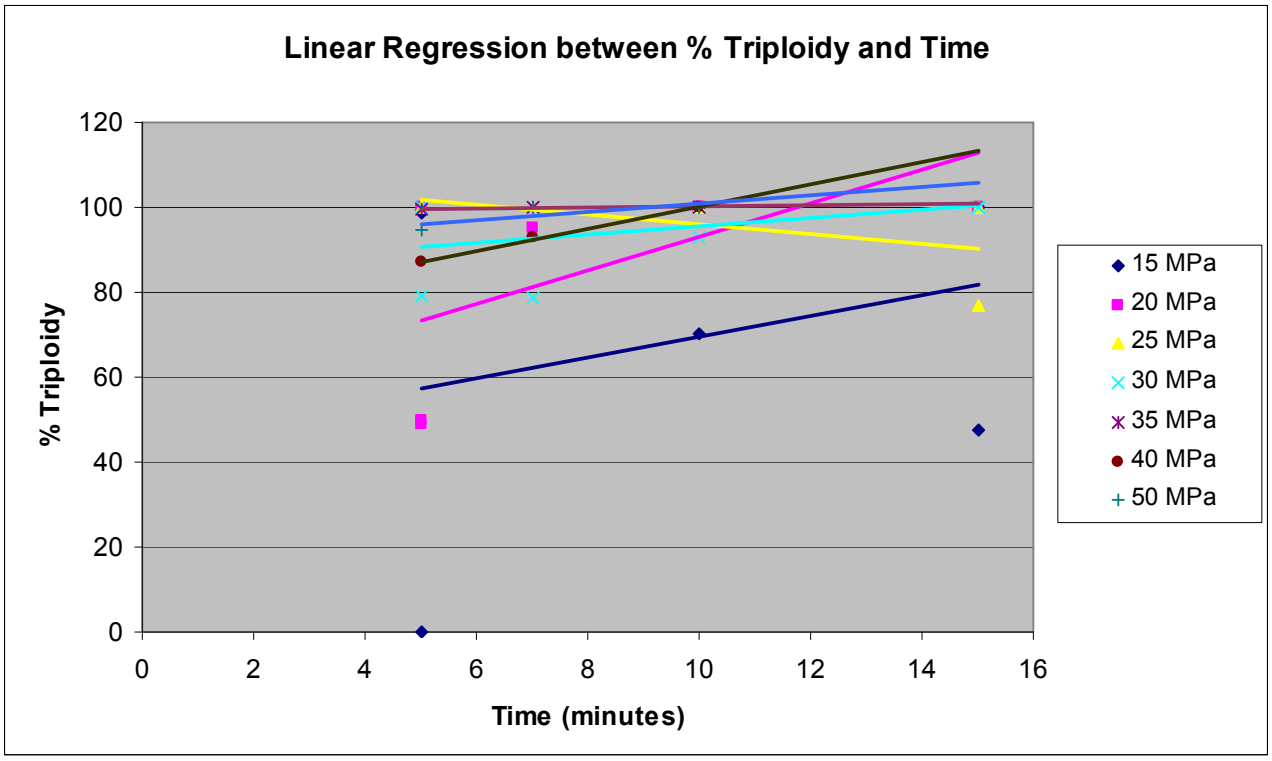

Figure 3.11 Linear regressions between percentage triploidy and treatment duration (time) for different pressures

Table 3.8 Equations for linear regressions between percentage triploidy and treatment duration at different pressures with standard errors, t-values and P-values for the slopes

Regression	Equation	Standard Error (slope)	T-value (slope)	P-value (slope)
15 MPa	y = 44.953 + 2.450(x)	2.105	1.16	0.2560
20 MPa	y = 53.529 + 3.964(x)	2.246	1.77	0.0902
25 MPa	y = 107.670 – 1.150(x)	2.105	-0.55	0.5899
30 MPa	y = 85.576 + 0.985(x)	1.869	0.53	0.6031
35 MPa	y = 98.888 + 0.122(x)	5.915	0.02	0.9838
40 MPa	y = 74.131 + 2.605(x)	5.915	0.44	0.6636
50 MPa	y = 90.737 + 1.013(x)	5.915	0.17	0.8654

For all seven linear regressions fitted (Figure 3.11, Table 3.8), the P-values (P>0.05) result in acceptance of the null hypothesis that the slope does not differ from 0, i.e. different time durations does not have a significant influence on the percentage triploidy at different fixed pressures. The slopes of lines 15, 20 and 40 MPa are steep and it can be observed that attainment of triploidy is variable, though with increasing effect, across different times at these pressures. The slopes of lines 25, 30, 35 and 50 MPa are more gradual, indicating that attainment of triploidy is less variable across different times at these pressures.

3.4.2 Survival

As before, the ANOVA as well as the Linear Regression according to the General Linear Model Procedure were conducted using SAS (SAS Institute Inc., 1996). Graphs were constructed with Microsoft Excel (Microsoft ® Excel 2002).

3.4.2.1 Analysis of Variance for testing the hypothesis of equal means for survival of the control and treated groups

Survival for the control larvae differed noticeably from that of the treated larvae. As presented in Figure 3.12, an overall mean of 89.086 ± 2.948 was obtained in terms of percentage of survival over all controls together with a coefficient of variance (CV) of 10.770. An overall mean of 43.584 ± 4.876 was obtained in terms of percentage of survival over all treatments together with a CV of. 70.915. The higher standard deviation and CV in the treated group indicate greater variation within the treated group than within the control in terms of percentage of survival. The results of a single factor ANOVA comparing the means of the control and the treatment groups are presented in Table 3.9. The null hypothesis of no difference between the mean survival for the control group and that of the treated group is rejected on the basis of a P-value of 1.32×10^{-5} (P<0.001). This implies that there is a statistically significant difference between the mean survival of the control group and the mean survival of the treated group.

Table 3.9 Results of a single factor Analysis of Variance for testing the hypothesis of equal means for control and treated groups

Source of variation	df	Sum of squares	Mean square	F	P-value
Between Groups	1	17764.510	17764.510	23.562	0.0000132
Within Groups	48	36189.235	753.942		
Total	49	53953.746			

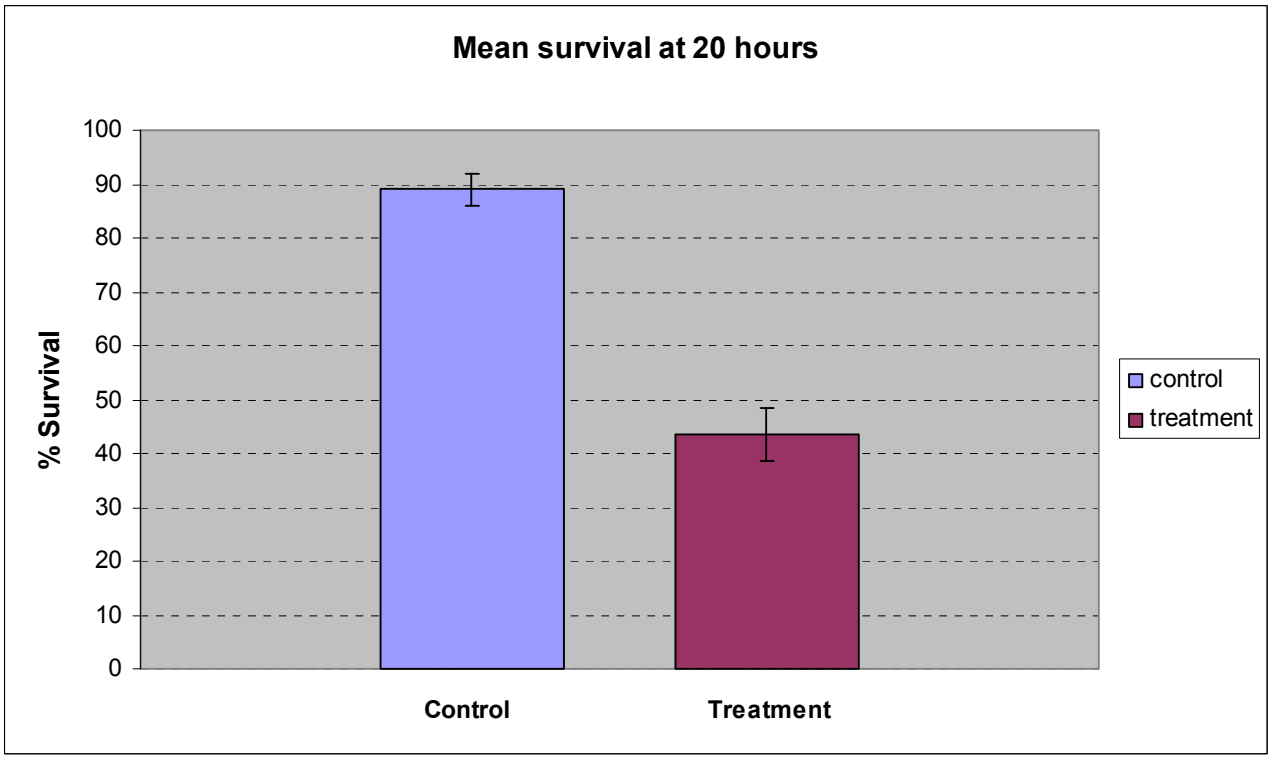

Figure 3.12 Mean larval survival at age 20 hours; control and overall treatment groups with Y-error bars indicating standard error of the mean

3.4.2.2 Analysis of Variance of the level of survival on the basis of different treatments

The results of an ANOVA for percentage of survival at age 20 hours on the basis of different treatments are presented in Table 3.10. The null hypothesis (H_o) of no difference in the level of survival on the basis of the different treatments is accepted on the basis of a P-value of 0.5561 (P>0.05). This implies that there was no statistical significance in the level of survival obtained from the different treatments. Separate *t*-tests (LSD) also

indicate that percentage larval survival of treated groups does not vary significantly with different pressures or with different times.

Table 3.10 Results of an Analysis of Variance for percentage of survival at age 20 hours on the basis of different treatments

Source	df	Sum of squares	Mean square	F	P
Treatment	24	21859.561	910.815	0.95	0.5561
Error	14	13373.891	955.278		
Corrected total	38	35233.452			

The results of the analysis of variance indicate that there are no significant differences in survival of larvae amongst the different time or pressure treatments. The data do, however report a significant difference between mean survival percentages for untreated and treated larvae.

3.4.2.3 Regression analysis of relationship between survival and pressure as variable at fixed durations of treatment

The linear regression between percentage survival and pressure treatments were fitted for each time period/duration. The R^2 value of 0.748 indicates that the linear regression model presents a proper fit to the data.

Table 3.11 Results of Linear Regression between percentage survival and pressure treatments with estimates of intercepts and slopes

Parameter		Estimate	Standard Error	T Value	P- value
Intercept	5 min	74.336	24.64	3.02	0.0051
	7 min	1.090	48.541	0.02	0.9822
	10 min	75.349	24.64	3.06	0.0046
	15 min	74.781	43.723	1.71	0.0972
Slope	5 min	-0.993	0.850	-1.17	0.2515
	7 min	0.990	1.334	0.74	0.4634
	10 min	-0.804	0.850	-0.95	0.3516
	15 min	-2.139	1.886	-1.13	0.2653

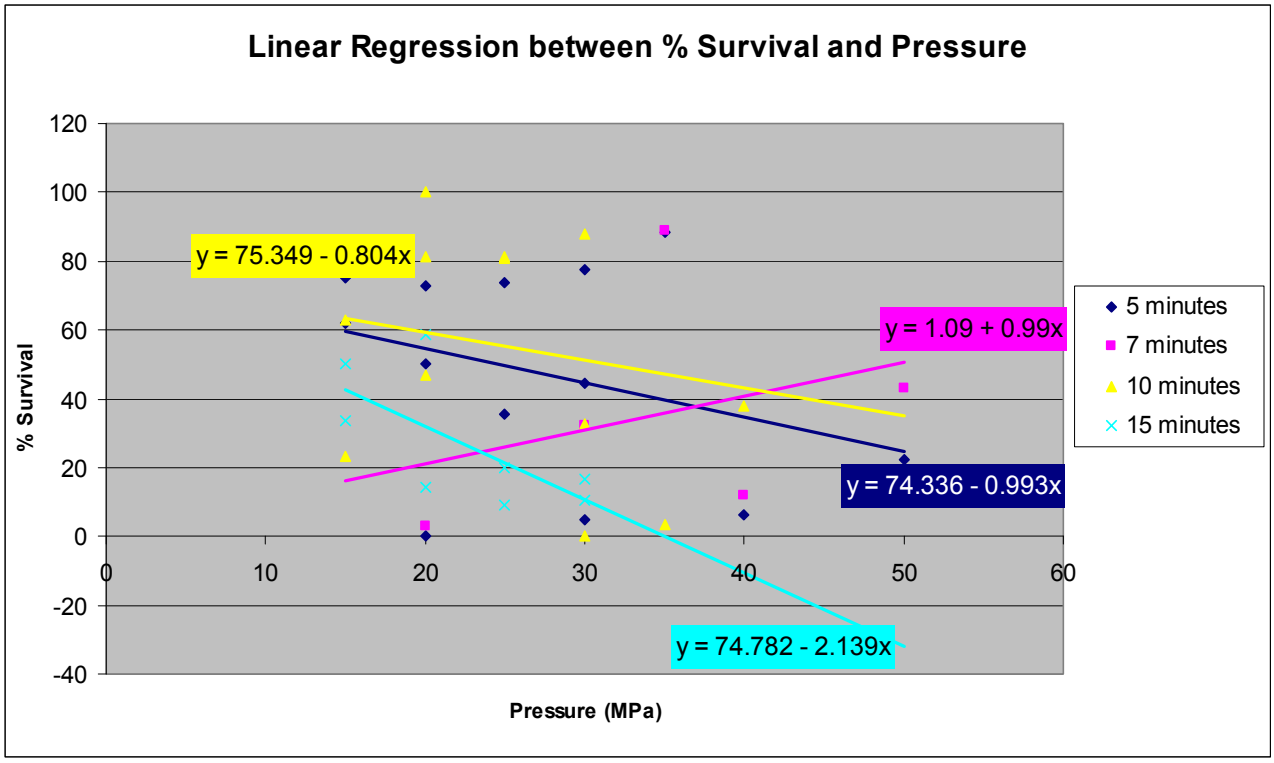

Figure 3.13 Linear regressions between percentage survival and pressure treatment for different time periods/durations

At all four time durations the linear regressions between percentage survival and pressure treatment indicate steep slopes with gradients varying between -2.139 and 0.99. This, along with the standard errors of the slopes, indicates that survival in treated larvae is variable across different pressure intensities for all the time periods. P-values between 0.2515 and 0.4634 (all $P>0.05$) result in acceptance of the null hypothesis that the slopes do not differ from zero, i.e. different pressure treatments does not have a significant influence on the percentage of survival at different treatment duration regimes. All the linear regressions, with the exception of time 7 minutes display a decrease in survival with increased pressure.

3.4.2.4 Regression analysis of relationship between survival and time as variable at fixed pressures

The linear regression between percentage survival and duration of treatment (time) was fitted for each pressure. The R^2 value of 0.794 indicates that the linear regression model presents a proper fit to the data.

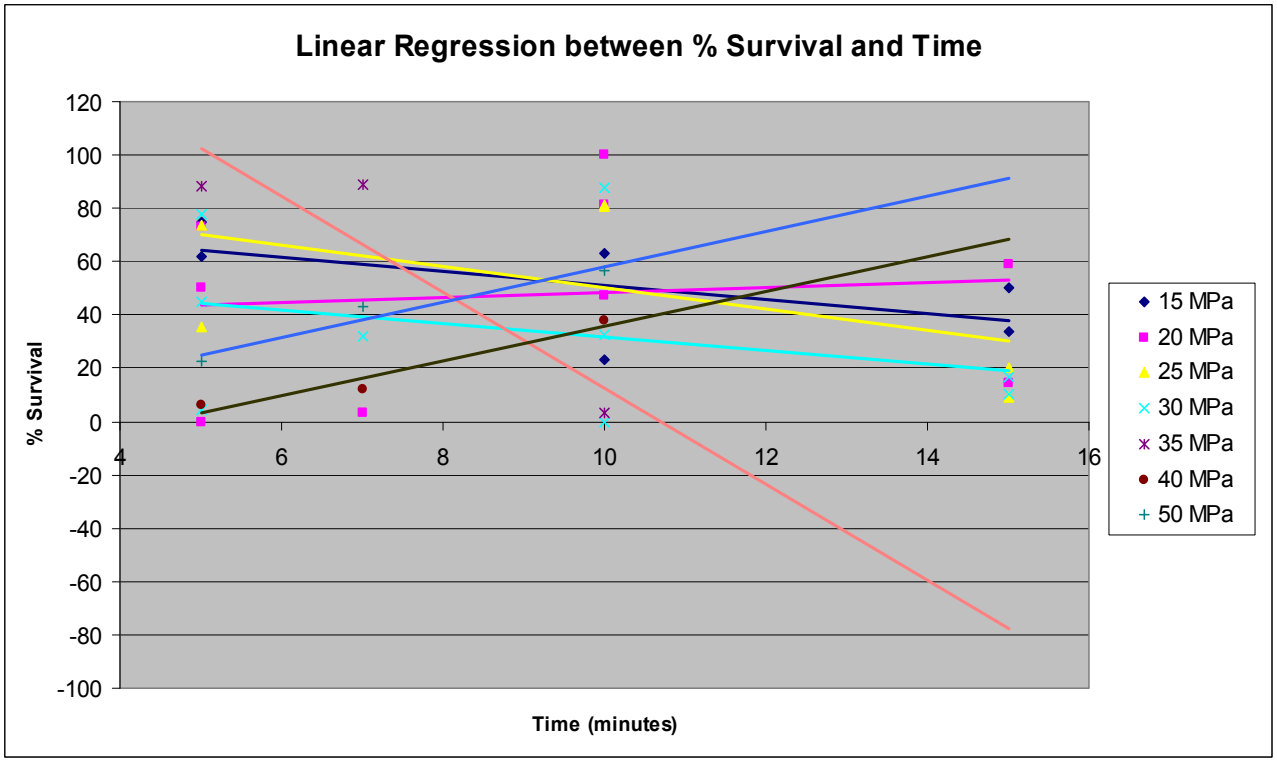

Figure 3.14 Linear regressions between percentage survival and treatment duration (time) for different pressures

Table 3.12 Equations for linear regressions between percentage survival and treatment duration (time) at different pressures with standard errors, t-values and P-values for the slopes

Regression	Equation	Standard Error (slope)	T-value (slope)	P-value (slope)
15 MPa	y = 77.670 – 2.656(x)	3.003	-0.88	0.3849
20 MPa	y = 38.784 + 0.956(x)	2.666	0.36	0.7229
25 MPa	y = 90.247 – 4.019(x)	3.003	-1.34	0.1929
30 MPa	y = 57.301 – 2.545(x)	2.666	-0.95	0.3489
35 MPa	y = 191.911 – 17.961(x)	8.438	-2.13	0.0433
40 MPa	y = -28.937 + 6.487(x)	8.438	0.77	0.4492
50 MPa	y = -7.800 + 6.600(x)	8.438	0.78	0.4414

At all seven pressures the linear regressions between percentage survival and time indicate steep slopes with gradients varying between -17.961 and 6.600. This, along with

the standard errors of the slopes, indicates that survival in treated larvae is variable across different time periods for all the pressure intensities. For all the linear regressions fitted, with the exclusion of the regression for pressure 35 MPa, the P-values ($P>0.05$) result in acceptance of the null hypothesis that the slope does not differ from zero, i.e. different time durations does not have a statistically significant influence on the percentage survival at different pressures. No clear trend or relationship could therefore be identified in relation to survival and the duration (time) of treatment.

The results for linear regression analysis between survival and pressure and between survival and time confirm the large extent of variation in survival within the treated group, similar to the results of the ANOVA. No clear trend could be established for the relationship between survival and pressure or survival and treatment duration (time).

3.5 Discussion

An aspect that was not fully accounted for in the experimental design of both experiments was the complete randomization in the application of treatments. Instead, a procedure of chronological application of treatments, with regard to duration and pressure levels, was applied. However, randomization was accounted for during the random allocation of eggs and sperm for fertilizations during each set of inductions. The respective treatment methods are also of an exact nature, in that specific pressures and timeframes were applied, reducing the effect of human and experimental error.

3.5.1 Levels of Triploidy

The possibility of triploid animals occurring in a natural population can not be excluded. The presence of triploid genotypes has been reported to occur in natural populations, though infrequently and considered as a numerical mutation of chromosomes (Guo and Allen, 1994; Yamamoto and Sugawara, 1988; Yang, Chen and Ting, 1998; Yang, Ting and Chen, 1998). Triploidy was, however, not detected in any of the untreated control groups in either of the experiments conducted during this study.

Another aspect to be considered when interpreting results of flow cytometry, as pointed out by Hand, Nell, Reid *et al.* (1999), is that the percentage of triploidy recorded may differ by five percent or more from the actual ploidy level when larvae are analyzed in groups. Such deviation could be caused by the presence of aneuploids, machine settings of the flow cytometer and varying cell numbers contributed by individual larvae to the analyzed samples.

The results from the two experiments gave an indication that triploidy can be successfully induced in *H. midae* over a wide range of pressure and time treatment combinations as confirmed by the high overall average and low standard deviation of triploidy observed across all treatment groups (89.8% ± 3.428%). The analysis of variance of percentage triploidy (Table 3.3) also confirms the null-hypothesis of no significant difference in the level of triploidy between the various treatments ($P>0.05$). On the basis of these results it can be concluded that no specific time and pressure treatment combinations can be excluded on the basis of suitability to induce triploidy in *H. midae*. The results also hold the advantage to industry that a range of time and pressure treatments can be considered for a procedural protocol, instead of a precise or particular time-pressure combination. The objective was to identify the ideal or optimal

range of treatments to be considered for application. Linear regression analysis was, therefore, conducted on the data to provide more information on the relationship between levels of triploidy and the respective time-pressure treatment combinations.

3.5.1.1 Regression analysis of relationship between levels of triploidy and pressure as variable at fixed durations of treatment

Figure 3.9 presents the linear regression between triploidy and pressure for each of four treatment durations/times. At all four durations/times the regressions display a trend of increased percentage triploidy with increased levels of pressure, all portraying positive gradient slopes.

A high a-value or intercept and low b-value or slope is advantageous as it indicates that high levels of triploidy can be maintained over a wide range of pressures at a given duration/time of treatment. Figure 3.9 and Table 3.4 indicate that the duration of 10 minutes maintained the highest intercept and lowest slope, followed respectively by 7, 15 and 5 minutes. In fact, for both the treatment at 7 and 10 minutes, the regressions comply with the hypothesis that the slope does not differ from zero ($P>0.05$). The steeper slopes obtained for treatment at 15 and 5 minutes are indicative of a much higher variability and thus a low probability to arrive at stable outputs of percentage triploidy when treating fertilized eggs for 5 or 15 minutes at any pressure.

From the results it is also clear that, for all the regressions, an increase in duration of treatment is associated with an increase in the level of triploidy. This is in agreement with the results reported in section 3.5.1.2.

A relatively high standard error was registered for slope in all four treatments as indicated in Table 3.4, i.e. 5 minutes: $b = 1.328 \pm 0.569$; 7 minutes: $b = 0.290 \pm 0.893$; 10 minutes: $b = 0.281 \pm 0.569$ and 15 minutes: $b = 1.492 \pm 1.281$. In order to reduce the standard error and increase the accuracy in the model, data for treatments 7 and 10 minutes were clustered, followed by a combined analysis. Data obtained from treatments 5 and 15 minutes were also clustered and treated in a similar manner. The reduction that was obtained in terms of standard errors and the narrowing of the 95 percent confidence intervals on the slopes are apparent from Tables 3.5 - 3.7 and Figure 3.10. The results from the combined analysis are therefore considered to represent a more reliable indication of *trends* pertaining to the effect of treatments for the induction of triploidy in *H. midae*.

As reported in Table 3.5, the high value of 90.325 ± 13.803 that was obtained for the intercept, together with the low value of 0.197 ± 0.442 for the slope of the 7+10 minute grouping, confirm with 95 percent confidence levels that high percentages of triploidy can be obtained over the full range of pressure treatments at these durations/times. In comparison, the estimated value of 53.854 ± 13.420 for the intercept and 1.174 ± 0.493 for the slope of the 5+15 minute grouping confirm a higher level of variation and lower predictability of levels of triploidy over the range of pressure treatments at these durations/times. In addition, the slope of the 7+10 minute line complies with the hypothesis that the slope does not differ from zero ($P>0.05$), while the slope of the 5+15 minute line ($P<0.05$) leads to rejection of this hypothesis.

On the basis of the above information and discussion, 7 to 10 minutes is recommended as the optimal duration of treatment for the induction of triploidy in *H. midae* by means of hydrostatic pressure, within the range of pressures tested.

3.5.1.2 Regression analysis of relationship between levels of triploidy and duration/time as variable at fixed pressures

The regression analysis between the level of triploidy and duration of treatment at various pressures indicates a general trend of increased percentage triploidy with increased duration/time (Figure 3.11, Table 3.8). The P-values ($P>0.05$), however, confirms the acceptance of the null hypothesis that the slopes do not differ significantly from 0, i.e. different durations of treatment do not have a significant influence on the percentage triploidy at the respective pressures. It is clear, however, from the data that the lower pressures (15 and 20 MPa) produce lower levels of triploidy and more variable results at the respective times/durations of treatment, as indicated by the respective intercepts and slopes reported in Figure 3.11 and Table 3.8. A similar trend of increased percentage of triploidy with increase in pressure corresponds to the results reported in section 3.5.1.1.

The results further indicate that the pressure range of 25 to 50 MPa maintain high levels of triploidy with good consistency over the full range of durations/time of treatments, to a lesser extent for pressure at 40 MPa (Table 3.8). Pressure treatment at 15 and 20 MPa is, however, not recommended for use on the basis of significantly lower levels of triploidy that are obtained as well as the lower consistency over the range of durations/time of treatment. Although high levels of triploidy are obtained with good consistency at pressures of 40 and 50 MPa, the application of such high pressures is also

not recommended due to the extreme biological strain on the larvae as well as physical strain on the hydraulic apparatus.

On the basis of the above information, 25 – 35 MPa is recommended as the optimal range of pressure treatment for the induction of triploidy in *H. midae* by means of hydrostatic pressure, irrespective of the treatment duration.

The percentages of triploidy attained over a wide range of treatment regimes are higher than reported for similar experiments on other species. Arai *et al*. (1986) reported 60 percent successful triploidy in hydrostatic pressure treated Pacific abalone, *H. discus hannai* (pressure intensity of 200kg/cm^2 = 19.6 MPa for five minutes). No other reports of hydrostatic pressure treatment in abalone could be found, although some studies are presently underway that may deliver comparable results (Grubert, Elliot and Ritar, 2003). For oysters, Chaiton and Allen (1985) reported a triploidy level of 57 percent for hydrostatic pressure treated *Crassostrea gigas* larvae at a pressure intensity of 6 000 – 8 000 pounds per square inch (psi) (41.4 - 55.2 MPa) for a duration of 10 minutes. Results by Peruzzi and Chaiton (2000) obtained for triploidy induction in fish, however, report 100 percent triploidy induction at shock intensities of 8 500 psi (58.6 MPa), at a treatment duration of 2 minutes in the European sea bass, *Dicentrarchus labrax* L. In Grass carp, hydrostatic pressure shocks are reported to result in 80 to 100 percent triploid fish when shock intensities of 7 000 to 8 000 psi (48.3 - 55.2 MPa) are applied for a treatment duration of up to 90 seconds (Rottmann, Shireman and Chapman, 1991).

Other induction methods did result in high percentages of triploidy in the abalone. Stepto and Cook (1998) reported 70.9 percent induced triploids using the Cytochalasin B chemical method of induction in the South African abalone, *H. midae*. In the same species, triploidy of 86.42 percent was attained using the elevated temperature shock (30˚C) method (Stepto, 1997). Arai *et al.* (1986) reported 70 - 80 percent triploidy when using cold shock (3˚C) and 60 - 80 percent when using elevated temperature shock (35˚C) in the Pacific abalone, *H. discus hannai*. Norris and Preston (2003) arrived at 96.4 percent triploidy with 6-DMAP chemical treatment in the Tropical abalone, *H. asinina*. Studies on triploid induction in the Australian blacklip abalone, *H. rubra* by Liu *et al.* (2004) resulted in 94 percent triploids using the 6-DMAP chemical method and 92 percent using the Cytochalasin B chemical method of induction.

3.5.2 Survival

Based on the overall results, survival was negatively influenced by the application of pressure treatments. A significant statistical difference is observed ($P<0.001$) between the mean survival of the control groups with that of the treated groups (Table 3.9). The difference between the mean survival of ~89 percent in untreated groups and the mean survival of ~44 percent in treated groups gives an indication of much lower survival of the treated groups. These results can be compared with those obtained by Yang, Chen and Ting (1998) in the small abalone, *H. diversicolor*, where significant differences in the percentage of normal embryos were found between treated groups and the control. Cold shock treated embryos exhibited survival levels of 12 to 15 percent at 6 hours after insemination in contrast to survival levels of 68 percent in the control groups. Stepto (1997) also reported significantly lower survival rates in ploidy treated groups of larvae of the South African abalone, *H. midae,* than in the control groups over a period of 120 hours. The results obtained in this study do not, however, agree with results obtained by Norris and Preston (2003) where chemical treatment with 6-DMAP had no significant effect on embryo fertilization and larval hatch rates in the tropical abalone, *H. asinina*.

An Analysis of Variance for percentage of survival at age 20 hours provided no evidence of statistically significant differences in the level of survival between the different treatments, i.e. time/duration and pressure. The null hypothesis of no differences in the level of survival between different treatments is therefore accepted ($P>0.05$). No distinction or recommendation can therefore be made in terms of the effect of specific time or pressure treatments, or combinations thereof, on the percentage of survival.

Linear regression analysis also revealed no indication of a trend between survival and pressure (Figure 3.13 and Table 3.11) or survival and duration of treatment (time) (Figure 3.14 and Table 3.12).

In viewing Figure 3.13, the regressions fitted between percentage survival and pressure for the durations 5, 10 and 15 minutes display negative gradient slopes indicative of a tendency of decreased percentage survival with increased pressure. Such a tendency is to be expected, as the larvae are subjected to extreme strain with increasing pressure. The regression fitted for time 7 minutes, however, does not conform to this observation in that it displays a positive gradient slope. The estimated slopes ranging from -2.139 to 0.99 (Table 3.11) indicates a high variability in percentage survival

across different pressure treatments over all the time periods. Although this general tendency of decreased percentage survival with increased pressure is expected, the P-values result in acceptance of the null hypothesis that the slopes do not differ from zero ($P>0.05$). The different pressure treatments are therefore considered not to have a significant influence on the percentage of survival at the various treatment durations/times.

The seven separate regressions fitted between survival and time for each pressure group presented in Figure 3.14 all display steep slopes with gradients ranging from -17.961 to 6.600 (Table 3.12). Large variation is observed in survival of treated larvae across different treatment durations at each of the pressure levels. As with the regressions between percentage survival and pressure, the P-values ($P>0.05$) for the regressions between survival and time form the basis of the acceptance of the null hypothesis that the slopes do not differ from zero. There is therefore no indication of a trend between the treatment duration/time and the percentage of survival at different pressure treatment levels.

The lower larval survival observed for the treated group may be explained by some factors that affect survival in larvae such as: gamete quality, ambient temperature, conditions during induction, water quality, bacterial contamination and stocking density of the rearing bins (Genade, Hirst and Smit, 1988; Hahn, 1989, Hand, Nell and Maguire,1998; Stepto, 1997; Yang, Chen and Ting, 1998). The randomized allocation of gametes to the treatment and control groups should negate the effect of gamete quality on the results. Care was taken to standardize environmental conditions that could affect survival. Both the control and the treated groups were kept at the same ambient water temperature and not exposed to extreme, harmful temperatures. Water quality was of a high standard such as used in the commercial production of abalone. Although care was taken in this regard, the larvae receiving treatment were handled more and put under severe strain in the pressure apparatus. More frequent handling could also be responsible for increased bacterial contamination, which is a major cause of larval mortality in hatcheries (Hahn, 1989). No record was taken of the stocking density and it is recommended that this is done in future experiments.

The procedure used for estimation of survival was insufficient to make clear inferences or predictions about later survival. It is suggested that survival is determined at

later larval stages also, such as 48 hours post fertilization, 5 to 7 days post fertilization (settlement stage) and throughout the 20 weeks following settlement (Liu *et al.*, 2004). According to Liu *et al.*, (2004), increased mortality before day 2 has been associated with larvae of the Blacklip abalone, *H. rubra,* treated with 6-DMAP. That is, before the larvae had completed 180˚ torsion of the foot mass. Beyond this stage, survival in the triploid treatment groups and the diploids was uniform, suggesting that day 2 larvae can be used as an early benchmark for evaluating the relative survival success of alternative triploidy induction protocols in *Haliotis rubra*. The settlement stage of larvae (day 5 in *H. midae*) has been described as the most critical stage in the life history of benthic organisms, such as abalone (Genade *et al*., 1988). Furthermore, the period between 8 and 16 days after settlement was found to be a critical period for the post-larvae (triploid and diploid) as this is when larvae change from endogenous feeding to exogenous feeding (Liu *et al.*, 2004). It is thus recommended to verify survival of larvae until after these stages.

3.6 Conclusion and Recommendations

The objectives of this project were to determine whether triploidy can be successfully induced in the South African abalone by using hydrostatic pressure shock to inhibit the extrusion of the second polar body, and to validate a successful method of identification of the induced triploids. The results of Chapter 3 provide confirmation of the efficiency of hydrostatic pressure shock to induce triploidy in the South African abalone over a wide range of pressure and durations of treatment. The use of flow cytometry as rapid and reliable method of identification of triploidy was validated by the results presented in Chapter 2.

The following recommendations could be considered in order to improve the experimental protocol during future studies of this nature:

1. The complete randomization of the experimental design, including the allocation of material and the application of treatments;
2. Alternative methods may be considered for verification of triploidy, such as chromosome counts (karyotyping);
3. Triploidy percentages should be verified at different developmental stages;
4. The stocking density of larvae in larval rearing bins should be recorded;
5. Survival should be determined at later larval stages, such as 48 hours post fertilization, 5 to 7 days post fertilization and throughout the 20 weeks following settlement;
6. Further studies examining the effects of triploidy on growth, metabolism, survival, and stress tolerance needs to be conducted to provide conclusive evidence of the advantages of triploidy.

“To be commercially beneficial, an induction technique must result in a high percentage of triploidy with a high percentage of survival.” (Yang, Chen and Ting, 1998). To comply with this recommendation and using the method of hydrostatic pressure induction, a spectrum of treatments where the best percentage triploidy results are attained and where both the biological material and apparatus receive minimum strain, appears to be a pressure in the range 25 - 35 MPa for a duration of 7 - 10 minutes. The proposal of a range has the advantage to the industry in that it provides for an easier procedural protocol, instead of a precise or particular time-pressure combination.

3.7 References

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CHAPTER 4: Final Conclusions and Recommendations

4.1 Background

Genetic improvement of cultivated abalone has become indispensable for the profitable farming and global competitiveness of the species. Ploidy manipulation can be employed to obtain sterility, which is a desirable trait in aquaculture organisms from a commercial point of view for numerous reasons.

The main reason entails faster growth because of higher energy availability for somatic growth in animals where energy is not required for gamete production. Studies on ploidy manipulation in the South African abalone to date have been limited despite the advantages it displays in other farmed abalone species elsewhere in the world.

In this study an attempt was made to bring the advantages associated with triploidy a step closer to the commercial production of the South African abalone by establishing a viable method of triploid induction and validation. An extensive literature study was conducted to assess the possibilities and to arrive at suitable methods for both induction and validation of triploidy. The objective of this study was to experiment with the hydrostatic pressure method of triploidy induction and flow cytometry as the method of validation on the South African abalone, *H. midae*, in order to produce offspring with the potential to exhibit advantages associated with functional sterility.

4.2 Conclusions

Based on the findings of this study it is recommended that the physical method of hydrostatic pressure is used for the commercial production of triploid abalone, instead of chemical methods. Hydrostatic pressure in the range 25 - 35 MPa for a duration of 7 - 10 minutes is recommended to provide consistent and high levels of triploidy. Flow cytometry is recommended as the method of verification of level of triploidy, and should be applied at progressive developmental stages due to the possibility of differential survival of genotypes. The impact of induction on the survival of triploid larvae should also be determined at progressive developmental stages.

The main conclusions drawn from the results obtained in this study are:

a. Flow cytometry is a reliable, fast and accurate, though expensive method for identification of triploidy in the South African abalone, *H. midae*;

b. Hydrostatic pressure is a valid method for induction of triploidy in the South African abalone, *H. midae*, resulting in consistent high percentages of triploidy in 48 h old larvae;
c. Survival in 20 h old larvae is negatively affected by hydrostatic pressure treatment;
d. These findings can be used as a basis for further investigation of alternative methods of production of triploids (e.g. tetraploid-diploid crosses) and to determine the effects of triploidy on growth, metabolism, survival, and stress tolerance in the resulting abalone;
e. A technical protocol has been developed in view of commercial application and is presented in the form of “Procedures for the Induction and Validation of Triploidy in the abalone, *H. midae*” (see Appendix 1)

Appendix 1:

Procedures for the Induction and Validation of Triploidy in the abalone, *Haliotis midae.*

A: Pressure Inductions

Spawning and fertilization

Female and male abalone are spawned according to the standard spawning procedure using hydrogen peroxide. The spawned gametes are kept in filtered seawater at a temperature of 17˚C until fertilization. All fertilizations should be conducted within two hours from spawning to assure gametes of the best possible quality. Eggs are siphoned from the bottom of the spawning basins into a clean glass beaker and sieved through a 400 – 500 µm mesh sieve to exclude debris onto a 100 µm mesh sieve. Excess sperm is added to the eggs and opportunity for fertilization is allowed for 10 minutes after which fertilized eggs are gently rinsed for 7 minutes. The fertilized eggs are now collected in a glass beaker in a concentrated form and transferred to the pressure apparatus for the pressure treatments.

Induction

The pressure apparatus consists of a stainless steel cylinder with a maximum volume of 150 ml. It is equipped with a copper headpiece fitted with rubber o-rings to fit tightly onto the cylinder, a pressure gauge, a hydraulic jack and a stainless steel frame.

The fertilized eggs are gently poured into the cylinder and air bubbles are removed by tapping the side of the cylinder with a nylon hammer. The headpiece is adjusted and the cylinder with headpiece placed on the stainless steel frame. Gentle pressure is applied with the hydraulic jack until water comes out of the pressure release opening in the headpiece. The pressure release lever is then closed. At 20 – 25 minutes after commencement of fertilization, pressure is applied to the fertilized eggs by pumping of the hydraulic jack. A pressure in the range of 25 – 35 MPa is applied for a duration of 7 - 10 minutes. Thereafter, pressure is instantly released and the fertilized, pressurized eggs transferred to an appropriate bin containing filtered seawater at 17˚C, where the larvae can be raised.

Flow diagram of Pressure Induction

Spawn female and male abalone

↓

Collect gametes separately (**within 2 hours** from spawning)

↓

Fertilize for **10 minutes**

↓

Rinse for **7 minutes**

↓

Transfer **150 ml** of concentrated, fertilized eggs to pressure apparatus

↓

Remove air bubbles from inside of cylinder by tapping

↓

Fit headpiece onto cylinder and insert up to indented line (pressure release lever open)

↓

Place cylinder on stainless steel frame

↓

Close pressure release lever

↓

At **20 – 25 minutes** after fertilization, apply pressure of **25 – 35 MPa** for **7 – 10 minutes**

↓

Instantly release pressure by depressurizing hydraulic jack

↓

Transfer eggs to rearing bin

B: Sample Collection and Preservation

The process of sample collection and preservation is very important since it influences the success of the flow cytometric analysis to follow. All collection procedures should be carried out under sterile conditions. Surfaces, hands and all equipment used should be cleaned and sterilized with alcohol.

Larvae of approximately 48 hours old are concentrated onto a 100 µm nytex screen and rinsed into a 50 ml falcon tube with ±10 ml 0.075 M KCl. The larvae are left in the KCl for 10 minutes and then again concentrated onto a 100 µm nytex screen and scooped into sterile 1.8 ml plastic cryovials. Ice cold Vindelov citrate buffer (1.5 ml) and DMSO

(150 µl) is then added before the samples are frozen at -20˚C. Samples may also be frozen at -80˚C if preservation for a longer period of time is required.

Flow diagram of Sample Collection and Preservation

Sterilize surfaces and equipment to be used; wash hands and wipe with alcohol

↓

Brush nytex screen with alcohol

↓

Scoop 100 µm nytex screen through bin with larvae until a layer of larvae is visible

↓

Rinse larvae from screen into glass beaker

↓

Concentrate larvae onto small area of nytex screen

↓

Rinse larvae from screen into 50 ml falcon tube with 10 – 20 ml 0.075 M KCl

↓

After 10 minutes in KCl, concentrate larvae on small area of nytex screen

↓

Scoop larvae from nytex screen with 1.8 ml sterile cryovial

↓

Add 1.5 ml Vindelov citrate buffer to larvae

↓

Add 150 µl DMSO shortly before freezing at -20˚C

C: Flow Cytometry

Sample preparation (nuclear isolation and staining)

Samples are taken from the -20˚C freezer and thawed at room temperature. From each sample, 50 µl of concentrated larvae is taken and 50 µl MPBS and 500 µl Propidium iodide (PI) staining solution is added to it. The suspension is then aspirated through a 1 ml syringe fitted with a 26½ G needle until no more clogging of the needle's tip occurs. The cell suspension is then filtered through a 25 µm nytex screen twice and centrifuged for 5 minutes at 5000 rpm. The supernatant is removed and the pellet of stained larval nuclei resuspended in 500 µl MPBS in a plastic eppendorf tube. The tube is wrapped in foil and kept on ice. The samples should be analyzed within 5 hours from staining.

Fluorescence microscopy

Before running the samples on the flow cytometer, they should first be examined on a fluorescence microscope. This is done to affirm that the samples are prepared satisfactorily for flow cytometry, so that minimum cell clumping and background staining is visible. Samples where brightly stained, red nuclei are visible are approved to be analyzed by flow cytometry.

Flow cytometry

The PI stained samples are analyzed on a Becton Dickinson FACSCalibur flow cytometer with a He/Ne laser (wavelength 488 nm). Prior to running, samples are vortexed and transferred to a 5 ml flow cytometry tube. For each sample, a minimum of 20 000 nuclei should be acquired at a rate of 100 to 250 nuclei per second to generate histograms. The following instrument settings are recommended to attain adequate histograms:

	Volt	**Amp**	**Mode**
P1	E00	3.00	lin
P2	500	1.00	lin
P3	320	1.00	log
P4	490	1.00	lin
P5	380	1.00	log
P6	-	1.00	lin
P7	-	3.94	lin
Threshold		20	
Compensation		0	

Flow diagram of Flow Cytometry

Sample preparation (nuclear isolation and staining)

Take samples from -20˚C freezer and thaw at room temperature

↓

Use 50 µl of concentrated larvae and add 50 µl MPBS and 500 µl Propidium Iodide (PI) staining solution to it

↓

Aspirate suspension through 1 ml syringe fitted with a 26½ G needle until no more clogging at tip of needle

↓

Filter cell suspension through 25 µm nytex screen twice

↓

Centrifuge for 5 minutes at 5000 rpm

↓

Remove supernatant and resuspend pellet of stained larval nuclei in 500 µl MPBS in a plastic eppendorf tube

↓

Wrap tube in foil and keep on ice

Fluorescence microscopy

Pipette a drop of the cell suspension onto a microscope slide and cover with a coverslip

↓

Examine under 20 x magnification on a fluorescence microscope

Flow cytometry

Vortex samples and transfer to a 5 ml flow cytometry tube

↓

Acquire ± 20 000 nuclei at a rate of 100 to 250 nuclei per second on the flow cytometer

Appendix 2:

Buffers and Staining Solutions

1. Vindeløv Citrate buffer (Vindeløv and Christensen, 1994)

Sucrose	85.50 g (250 mM)
Trisodium citrate, $2H_2O$	11.76 g (40 mM)
Dissolve in distilled water	± 800 ml
DMSO	50 ml
Distilled water is added to a total volume of	1000 ml
pH is adjusted to	7.6

2. MPBS (Marine phosphate buffered saline) (Allen, 1983)

NaCl	11.00 g/l
KCl	0.20 g/l
$Na_2HPO_4.2H_2O$	1.15 g/l
KH_2PO_4	0.20 g/l

3. PI staining solution (per sample) (Norris and Preston, 2003)

MPBS

0,1% Triton X-100

0.2 $mg.ml^{-1}$ RNAse A

0.01 $mg.ml^{-1}$ Propidium Iodide

4. Vindeløv nuclear isolation solutions (Vindeløv and Christensen, 1994)

4.1 Stock solution

Trisodium citrate, $2H_2O$	1000 mg (3.4 mM)
Nonidet-P40	1000 µl (0.1% v/v)
Spermine tetrahydrochloride	522 mg (1.5 mM)
Tris	61 mg (0.5 mM)
Distilled water added to a volume of	1000 ml

4.2 Solution A

Stock solution	1000 ml
Trypsin	30 mg
pH adjusted to	7.6

4.3 Solution B

Stock solution	1000 ml
Trypsin inhibitor	500 mg
Ribonuclease A	100 mg
pH adjusted to	7.6

4.4 Solution C

Stock solution	1000 ml
Propidium iodide	416 mg
Spermine tetrahydrochloride	1160 mg
pH adjusted to	7.6