

**A Comparative Analysis of Growth Traits in Triploid and Diploid  
Genotypes of the South African abalone, *Haliotis midae*.**

by  
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## Abstract

Abalone production is the largest financial contributor to aquaculture in South Africa and practically all of the abalone produced is exported to Asia. This means that the product must be globally competitive and many technologies have been applied to this cause. One that specifically shows great promise for bivalve mollusc production is triploidy; more precisely, sterility due to the induction of aneuploidy.

Under normal maturation, energy is diverted from somatic growth through sexual maturation, therefore inhibiting or retarding gametogenesis through a process such as aneuploidy is expected to increase growth and decrease the time to marketing.

Two studies preceding this one investigated the induction of triploidy through hydrostatic shock (De Beer, 2004) and the comparative growth rate of triploid genotypes from 8 to 24 months, prior to the onset of sexual maturation (Schoonbee, 2008). During this comparative growth stage, no convincing statistical evidence of faster growth or of seasonal environmental effects could be obtained.

It was recommended that growth between triploid and diploid variants be compared during the age period when sexual maturity becomes a factor to determine whether triploidy in *Haliotis midae* is a useful biotechnological tool to improve biological productivity and global competitiveness of the abalone industry.

The growth measured as shell length and wet weight in the period from 29 to 62 months showed a statistically significant difference in mean weight and mean length with diploids showing a superior growth rate compared to their triploid siblings. This difference of 1.99 mm and 5.13 g was however not perceived as being commercially significant.

Important production parameters including canning yield percentage and gonadosomatic index were also measured during this trial. For both these parameters, the triploid genotype showed statistically and commercially significant improvement of 10.68% increased canning yield and 28.42% reduction in gonadosomatic index when compared to their diploid counterparts.

Triploid abalone was found to be not completely sterile; gametes and even mature gonads were observed in some instances. Even though complete sterility was not achieved there appeared to be a retarded gonadosomatic development in triploid

variants. The delay in sexual maturation, together with the improvement in canning yield, may justify triploidy's commercial application, despite its reduced growth rate.

## Opsomming

Perlemoen produksie lewer die grootste finansiële bydra tot akwakultuur in Suid Afrika en feitlik al die Perlemoen word uitgevoer na Asië. Dit beteken dat die produk moet kompeteer op die wêreld mark en verskeie tegnologieë word reeds aangewend vir die spesifieke doel. Een so tegnologie wat potensiaal toon ten opsigte van akwakultuur produksie is triploëdie; meer spesifiek, sterieliteit veroorsaak deur aneuploëdie induksie.

Onder normale volwassewording, word energie weggeneem van somatiese groei wanneer geslagsrypheid intree en daarom kan groeitempo verhoog word deur gametogenese te inhibeer of te vertraag deur 'n proses soos aneuploëdie en, word 'n korter tydperk benodig om bemarkingsgrootte te bereik.

Twee voorafgaande studies het gehandel oor die induksie van triploëdie deur hidrostatische druk skok (De Beer, 2004) en die vergelykende groeitempo van triploëde genotipes vanaf ouderdom 8 tot 24 maande (Schoonbee, 2008) alvorens geslagsrypheid intree. Tydens hierdie vergelykende groeifase kon geen statisties betekenisvolle aanduidings van vinniger groei of seisoenale omgewingseffekte aangetoon word nie.

Die studie handel vervolgens oor die uitbreiding van die vergelykende groeistudies tussen triploëde en diploëde genotipes tot 'n ouderdom van 62 maande wat die intrede van geslagsrypheid insluit, ten einde te bepaal of die induksie van triploëdie in *Halotis midae* voordele inhou ten opsigte van produksiedoeltreffendheid en mededingendheid op wêreldmarkte.

Groei gemeet in terme van skulplengte en lewende massa oor die tydperk van 29 tot 62 maande het statisties betekenisvolle verskille getoon in gemiddelde massa en lengte van diploëde genotipes bo die van triploëde verwante individue. Die verskille van 1.99 mm en 5.13 g kan egter nie as kommersieel betekenisvol beskou word nie.

Belangrike produksie eienskappe waaronder persentasie opbrengs van eindproduk en die gonadosomatiese indeks is ook bepaal. Vir beide die produksie eienskappe het die triploëde genotipe 'n statisties sowel as kommersieel betekenisvolle verbetering van

10.68% getoon vir opbrengs en 28.42% verlaging in gonadosomatiese indeks in vergelyking met die diploïede genotipe.

Triploïede genotipes was nie volledig steriel nie, gegewe die aanwesigheid van gonades en gamete in sommige individue. Selfs al is totale steriliteit nie bereik nie, het dit wel voorgekom asof daar 'n vertraging in gonadosomatiese ontwikkeling plaasgevind het in triploïede genotipes. Die vertraging in geslagsrypheid tesame met die verhoogde persentasie opbrengs van die eindproduk hou voordele in bo die andersins effense stadiger groei van triploïede genotipes.

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# 1. Rationale

## 1.1 World aquaculture

Aquaculture is the farming of aquatic organisms (fish, molluscs, crustaceans, aquatic plants, etc.). Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated (FAO, 2003).

Aquaculture started more than 4 500 years ago in China. Aquaculture has since developed into a dynamic and technologically driven industry with global production that increased from less than a million metric tonnes in the 1950's to more than 55 million metric tonnes in 2009 (FAO, 2010).

China is the world leader in aquaculture with 67.3% of production. Wild fisheries has maintained a relatively constant rate of harvest of 90 to 100 million metric tonnes per year for the past 30 years while aquaculture, in contrast, has grown by an average of 8.8% per year over this period (Subasinghe, *et al.*, 2009). In 2009, the total world fisheries production was an estimated 145 100 000 metric tonnes with aquaculture contributing an additional 55 100 000 metric tonnes (FAO, 2010). Aquaculture is recognized as the fastest growing food industry and is contributing almost 50% of fish consumed by man (Subasinghe, *et al.*, 2009).

## 1.2 Aquaculture in South Africa

Aquaculture in South Africa is a recent development made up of small, medium and large, often vertically integrated, operations. In 2008, the total South African aquaculture production was 3 664 metric tonnes with a farm gate value of R327 million (Britz, *et al.*, 2009). The main cultured species are abalone, trout, oysters and mussels with a focus on the introduction of candidate species including prawns, kob, yellowtail, tuna and seaweed.

Aquaculture production measured in tonnage has grown at an average rate of 7.8% for the period of 2005 to 2008 while total value has increased by 32% per annum over the same period. The growth in marine aquaculture in the last few years is attributed mainly to the production of the high value abalone, *Haliotis midae*, which increased from 662

metric tonnes in 2005 to 934 metric tonnes in 2008. Aquaculture development in South Africa focus mainly in the production of high valued species such as abalone, oyster and trout (Britz, *et al.*, 2009) and it is expected to remain so over the short to medium term.

### **1.3 Abalone production in the world**

The commercial farming of abalone began in earnest in the 1990's in Japan and China because of a decline in landings from wild fisheries (Hahn, 1989). Abalone farming is now practised in countries on most of the major continents of the world. These include the USA, Mexico, South Africa, Australia, New Zealand, Korea, Taiwan, Ireland, and Iceland (Troell, *et al.*, 2006). Of these countries China is the world leader with a total production of 4 500 metric tonnes produced by over 300 farms (Gordon and Cook, 2004).

Globally fisheries for abalone are based on mainly 14 commercially important species (Lindberg, 1992; Godfrey, 2003). The global production of abalone has increased from 22 600 metric tonnes in 2002 to 38 600 metric tonnes in 2007 (Fishtech, 2009). Global production is made up of farmed, fished (also referred to as captured) and poached abalone (Gordon and Cook, 2004). In 2008 the production of farmed abalone was estimated at 30 760 metric tonnes. This farmed production contributes about 69% of the global production of 44 510 metric tonnes (FAO, 2010). Poaching is, however, a major problem and has resulted in the overexploitation of resources and the collapse of abalone fisheries in many countries (Rogers-Bennett, *et al.*, 2002). Poaching was estimated at a level of 5 300 metric tonnes in 2008 (Cook and Gordon, 2010).

### **1.4 Abalone production in South Africa**

A total of six haliotid species occur in southern African waters with only *Haliotis midae* being commercially exploited through farming and fisheries (Tarr, 1995; Sales and Britz, 2001). Abalone farming in South Africa started in 1981 when captured broodstock was successfully spawned for the first time to produce spat, whilst the fishery has existed since 1949 (Sales and Britz, 2001).

Commercial abalone fisheries have declined dramatically over the past 30 years from a level of total allowable catches in excess of 2 700 metric tonnes in the 1970's to a mere

125 tonnes in 2006. This decline as a result of over-exploitation of the wild resource through over fishing and illegal poaching, has culminated in the closure of the commercial abalone fishery in 2007 to 2010 with the emphasis shifting towards commercial farming (Schoonbee, 2008).

Over-exploitation of wild abalone stocks, high market prices, suitable coastal habitat and available infrastructure has led to the rapid expansion of abalone farming in South Africa from the mid 1990's. South Africa has since developed into the largest producer of farmed abalone outside of Asia (Troell, *et al.*, 2006; FAO, 2009). This expansion was also supported by the development of globally competitive abalone culture technology through collaboration between industry and various research institutions (Sales and Britz, 2001).

There are 12 abalone farms in South Africa (9 in the Western Cape, 1 in the Eastern Cape and 2 in the Northern Cape) where animals are grown in tanks on land. Commercial culture of the abalone *H. midae* has become the most valued aquaculture species in South Africa and in 2008 it was valued at R268 million. This represents 81% of the rand value of aquaculture in South Africa. The total production was 934 metric tonnes in 2008 (Britz, *et al.*, 2009). The growth of the abalone industry in South Africa is expected to continue over the medium to long term.

## **1.5 Biotechnology in abalone farming in South Africa**

The South African abalone culture technology has gained from technology transfer from foreign industries including California, New Zealand and Australia, innovation by local industry and collaboration between research institutions and industry (Sales and Britz, 2001; Troell, *et al.*, 2006; Schoonbee, 2008). Most of the developments of abalone culture technologies to date were based on development and improvement of husbandry and management systems as well as improving feeding technologies (Schoonbee, 2008).

Genetic biotechnology has recently been introduced as a further means to develop globally competitive culture technologies. These genetic technologies include polyploidization, hybridization and selection for genetic improvement of biological productivity in abalone farming. Ploidy manipulation, as applied in various other commercial shellfish species, was also identified as a mean to improve growth rates of *H. midae* (De Beer, 2004).

## 1.6 The use of triploidy in abalone farming in South Africa

In the wild, *H. midae* can take up to 7 years to reach sexual maturity. Maturity is linked to body size, and in *H. midae*, it occurs from 80 to 105 mm shell length. Mostly due to improved artificial feeds and grading procedures, this size is attained within 3 to 4 years in commercial farming operations. This is also the preferred marketing size. This is a much faster growth rate than typically found in the wild, but it remains a long grow-out period before the animals are suitable for marketing (Sales and Britz, 2001).

During this long production period, most abalone achieve sexual maturity on the farm before it is marketed. At the onset of sexual maturation less energy is available to sustain somatic growth, hence a reduced growth performance (measured as shell length and muscle weight increase) relative to feed intake, manifesting in an increased, poorer feed conversion ratio in abalone farming (Schoonbee, 2008).

Sexual maturity during the production period is also unwanted due to random spawning events. Triggers, such as stress due to environmental factors, cause the abalone to spawn which has a negative impact on animal condition and water quality. The largest market for South African abalone is live exports to Asia, during which the maintenance of water quality is very important, which can be negatively affected by spawning of animals in transit. Spawning also lowers the general quality of the product (Sales and Britz, 2001).

Abalone for production is spawned from a small population or brood stock consisting of only a few individuals. This may result in a very large farm population of fairly homogenous genotypes that may have a negative effect on the surrounding natural population in the event of uncontrolled release of farmed animals. Furthermore, water used in the production units are circulated and returned to the ocean as effluent (personal communication, Lize Schoonbee). If individuals spawn randomly, fertilized larvae can potentially be released into the wild, which may influence the genetic diversity of the wild population and compounds biosecurity considerations.

A new abalone farming strategy in South Africa is that of seabed ranching. This requires the artificial spawning and rearing of juveniles to a suitable size before releasing the animals into the wild. They are then left to grow naturally and are later harvested. This may also result in important biodiversity problems (Katsuo, 2003).

These aspects emphasize the benefits that may arise from triploidy and the related sterility. It may lead to improved growth rates in triploids due to energy relocation from reproductive growth to somatic growth, while the reduced spawning activity might have a positive impact on meat quality (Schoonbee, 2008; Liu, *et al.*, 2009). Further benefits of sterility include improved biosecurity as well as protecting intellectual property as in the case with genetically improved broodstock.

Triploidy has been successfully introduced in the oyster industry, with commercialization of the technology in North America since the early 1980's, which has promoted the application of triploidy in other mollusc species, including abalone (Schoonbee, 2008). Although there are exceptions (Schoonbee, 2008, Liu, *et al.*, 2009), improved growth rates have been widely reported for triploid molluscs (Stepto, 1997; Zhang, *et al.*, 1998; Liu, *et al.*, 2004), although complete sterility have not yet been confirmed. Variable growth responses reported for studies on triploid molluscs are probably due to a retarded gonadal development or abnormal gametogenesis that has been commonly observed (Schoonbee, 2008; Liu, *et al.*, 2009).

The advantages of triploidy include; improved growth rate, higher percentage product yield, improved meat quality, biosecurity advantages and improved management options (Schoonbee, 2008).

An initiative by I&J Abalone Culture Division and Stellenbosch University led to the induction of triploidy in 2002 to investigate the effect on the growth performance and reproductive status of *H. midae* (De Beer, 2004; Schoonbee, 2008). This project was continued from 2006 by an industry consortium in partnership with the SA Innovation Fund Trust.

De Beer (2004) developed a protocol to induce triploidy through the application of hydrostatic pressure (Figure 1.1) that prevented the extrusion of polar body II after fertilization; a technique that produced triploidy levels in the range of 95 – 100%.

Schoonbee (2008) investigated the effect of this pressure induction on the survival and growth rate of



**Figure 1-1** The pressure induction apparatus

triploid *H. midae* through all the stages of development from fertilization to an age of 24 months. She reported no significant difference in growth rate between diploid and triploid genotypes, based on shell length and wet weight, and recommended that the comparative growth analysis be continued beyond the age of sexual maturation as to when the expected advantages of sterility should come into effect (Schoonbee, 2008).

The main objectives of this study is to:

- Evaluate the growth rate of diploid and triploid genotypes of the abalone, *H. midae*, through the onset of sexual maturation (months 29 to 62)
- Assess the effect of triploidy on important production parameters such as canning yield and gonadal development

## 1.7 Conclusion

Aquaculture development in South Africa is small when compared to the rest of the world but the local abalone farming industry has developed into a significant global player. Nearly all of the abalone farmed in South Africa are exported to Asia, which implies that the product produced in South Africa must be globally competitive in terms of cost and quality. Various technologies have been applied to contribute to this cause, including that the use of triploidy.

A previous study by De Beer (2004) developed a protocol for the induction of triploidy in the South African abalone through hydrostatic shock upon which Schoonbee (2008) compared survival and growth rate between diploid and triploid genotypes through the early life stages before the onset of sexual maturity.

The current study continued with the comparison of growth between triploid and diploid genotypes throughout the remainder of the production cycle, up to life stages of sexual maturation. It is the main objective of this study to investigate the perceived advantages of triploidy in the abalone, *H. midae*, and to determine whether triploidy can contribute towards the competitiveness of the SA abalone industry.



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## 2. Literature Review

### 2.1 Biological aspects related to *Haliotis midae*

#### 2.1.1 General classification

The phylum Mollusca is a large group, which includes approximately 50 000 living species with great diversity that includes clams, scallops, sea slugs, abalone, octopuses and squid (De Beer, 2004; FishTech, 2009). Abalone, or Haliotids, is from the family *Haliotidae* in the class Gastropoda and a summary of their taxonomic classification is presented in Table 2.1.

Gastropods are molluscs with a discrete head with eyes and tentacles, a broad and flattened foot and a mouth with radula. The body is asymmetrical with a spiral shell covering the entire animal (Muller, 1986).

The genus *Haliotis* can be found in most of the continents' rocky coastlines and islands in the Pacific, Indian and Atlantic Oceans. The genus includes 56 recognised species (Geiger, 2000) that occur in tropical and temperate regions of the World and they represent the oldest and most primitive group of prosobranch gastropods (Brown, 1993, De Beer, 2004; Schoonbee, 2008).

**Table 2-1** Taxonomic classification of the abalone *Haliotis midae* (Hahn, 1989)

Phylum	<i>Mollusca</i>
Class	<i>Gastropoda</i>
Subclass	<i>Prosobranchia</i>
Order	<i>Archaeogastropoda</i>
Family	<i>Haliotidae</i>
Genus	<i>Haliotis</i>
Species	<i>midae</i>

Six indigenous species of abalone occur in the coastal waters of South Africa, the geographic distribution of which is presented in Table 2.2. Of these species, only *H. midae* is commercially exploited and farmed in South Africa.

**Table 2-2** *Haliotis* species and their occurrence in South Africa (Muller, 1986)

Species	Distribution
<i>H. midae</i>	Saldanha to Port St. Johns
<i>H. parva</i>	Cape Town to East London
<i>H. spadicea</i>	Cape Town to Sodwana
<i>H. alfredensis</i>	Port Alfred to Port St. Johns
<i>H. queketti</i>	East London to Durban
<i>H. pustulata</i>	North of Sodwana

*Haliotis midae*, or Perlemoen as it is locally called, is the largest of the local abalone species growing to over 200 mm in size (Sales and Britz, 2001). They occur off rocky shores, from the low tide mark to a depth of about 10 meters, along much of the coast from around Dwesa on the East coast to St. Helena Bay on the west coast of South Africa (Muller, 1986). Over-exploitation and rampant poaching have impacted severely on South African wild abalone stocks and future abalone production of the species will be predominantly dependent on aquaculture.

### 2.1.2 Applied anatomy of *Haliotis midae*

Shell condition is a good indicator of the general health status of an abalone and the condition of the shell will reflect the history relating to diet, environmental conditions and growth rate of that abalone (Schoonbee, 2008).

The shell of *H. midae* often has a reddish-brown colour (Figure 2.1) but this can be highly variable in cultivated abalone based on the diet. In the wild, the shell is often covered by marine growths, but this tends to be less of a problem in farmed individuals (Muller, 1986; Schoonbee, 2008).

Veliger larvae (15 to 48 hours after fertilization) form spiral shells called protoconchs, which serves as the apex of the adult shell (Bevelander, 1988). The shell is perforated

by a row of respiratory holes. These are used to excrete water from the gills, waste products and reproductive material.

Growth occurs by depositing new shell material on the growing edge of the shell and as this continues through adulthood, the older respiratory pores close up in succession (Muller, 1986; Bevelander, 1988; Schoonbee, 2008). *Haliotis midae* will grow to an average of 200 mm with the shell continuing to thicken through its life (Newman, 1968; Bevelander, 1988; Hahn, 1989; Schoonbee, 2008).

Farmed abalone will compete for food if they are housed at high density and this will result



**Figure 2-1** Ventral and dorsal view of *Haliotis midae* shell

in stacking and shell breakages in weak spots (Tarr, 1995; Huchette, *et al.*, 2003; Schoonbee, 2008). In abalone, the shell is the most prominent feature and appreciated for its goniochromism mother of pearl inner layer (FishTech, 2009).

Abalone is cultivated for its edible muscular foot, which can make up 30% of its mass. The foot provides suction that allows the abalone to attach tightly to rocky surfaces in its intertidal habitat. A column of muscles called the adductor, attaches the abalone to its shell.

The foot is surrounded by epipodia and the mantle. The shell also covers the anterior head. The epipodia are sensory tentacles and are also used in identifying species (De Beer, 2004; FishTech, 2009).

The foot is the only energy deposit available to the animal and is usually pale cream to mottled light brown in colour but this can also vary according to the diet (Muller, 1986; Schoonbee, 2008).

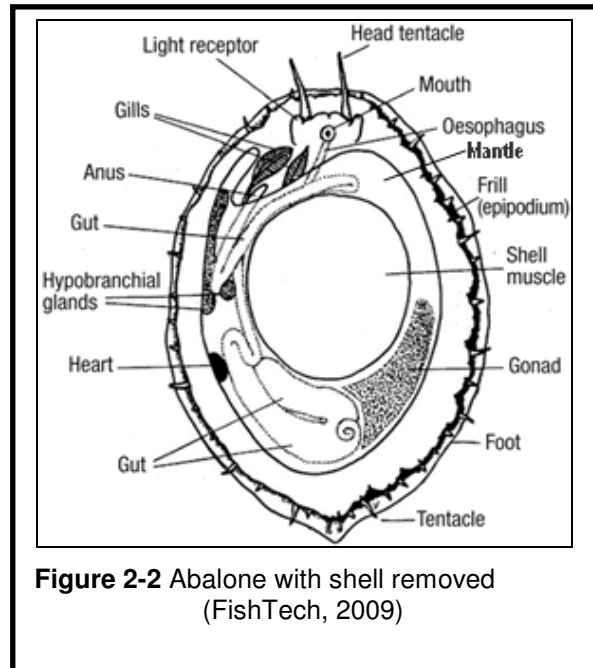
The internal organs of the abalone (Figure 2.2) are arranged under the shell, around the muscular foot. Abalone has a single gonad (dioecious) that encircles the hepatic gland (digestive gland) and when sexually mature can make up 15 – 20% of the soft-body mass. The gonad, also referred to as the conical appendage along with the intestine, is visible from the ventral side in mature *H. midae* individuals with the ovary displaying a greenish colour and the testis, a cream colour.

The gonad lies on the opposite side of the respiratory pores and towards the back (posterior) end of the abalone. After spawning the gonads decreases in size (Newman, 1967; Purchon, 1977; Hahn, 1989; Henry, 1995). The gonad consist of a large lumen with germinal epithelium attached by connective tissue and is well supplied with blood vessels.

The testis produces spermatocytes that develop into spermatids. Sperm that are six micrometers long, excluding the tail, is formed from the spermatids. In the ovary, the lumen is filled with eggs embedded in a gelatinous matrix. The eggs can be up to 200 micrometers in diameter (Newman, 1967).

### 2.1.3 Reproduction

In the wild abalone are sexually mature after three to five years and a size of 80 - 100 mm shell length, but this is dependent on water temperature, with *H. midae* on the warmer East Coast of Africa maturing earlier than individuals on the colder West Coast (Barkai and Griffiths, 1988; Henry, 1995; Tarr, 1995; Sales and Britz, 2001). An adult is defined as being sexually mature when it has spermatozoa or primary oocytes (Hahn, 1989). Most wild populations spawn twice per annum, normally in spring and autumn (Newman, 1967).



**Figure 2-2** Abalone with shell removed (FishTech, 2009)

Halitids have a primitive reproduction strategy that is limited to the emission of large numbers of gametes into the environment. It provides no protection or care to the developing gametes (Purchon, 1977). Because of mass spawning events, it is important that individuals within the population have reproductive cycles that are in phase. Higher fertilization success is accomplished if individuals spawn in close succession to each other (Hahn, 1989).

Spawning can be artificially induced by using chemical and physical treatments including UV-light, ozone treatment using hydrogen peroxide, pH alterations and water temperature manipulation (Fallu, 1991). Egg production in abalone is in the order of several million per individual and they have very high fecundity. This is a function of ovary volume and a linear relationship has been established between fecundity and animal weight (Newman, 1967).

## 2.2 Triploidy in shellfish

Research on polyploidy in shellfish began in the 1980's with the main objective to develop technology to produce commercial quantities of sterile oysters of the species *Crassostrea virginica* (Utting, 1995). In 1985 the first triploid Pacific oysters (*Crassostrea gigas*) was commercially produced in North America (Allen and Guo, 1996; Nell, 2002). Since then triploidy has been successfully induced in various other species of molluscs, including oysters (Garnier-Gere, *et al.*, 2002; Davis, 2004), clams (Liang and Utting, 1994), mussels (Brake, *et al.*, 2004), scallops (Tabarini, 1984; Yang, *et al.*, 2000) and some abalone species (Zhang, *et al.*, 1998; Elliot, *et al.*, 2004; Liu, *et al.*, 2004).

During sexual reproduction in marine molluscs, germ cells undergo two maturation divisions through the process of meiosis before becoming gametes (Guo *et al.*, 1992). Triploidy can be induced in fertilized eggs during two occasions, Meiosis I or Meiosis II, by suppressing the formation of the polar bodies, with the result of retaining an additional set of chromosomes in the cell nucleus (Boudry, *et al.*, 1998; Gérard, *et al.*, 1999).

In most organisms, gametogenesis continues throughout the two nuclear divisions completing all the steps of meiosis, including the release of polar bodies I and II, before fertilization occurs. In molluscs, however, there is a delay in meiosis and the eggs are arrested at prophase of Meiosis I, followed by the two divisions of Meiosis I and II that occurs after fertilization. The release of polar bodies I and II therefore occur after fertilization and this provides the specific opportunity to manipulate the number of sets of chromosomes through the retention of the polar bodies (Guo, *et al.*, 1992; Hahn, 1989). Sterility arises in the adult triploids because the triplicate sets of homologous chromosomes in the germ cells cannot synapse during meiosis and gametes cannot be produced (Beaumont and Fairbrother, 1991).

Triploid advantage is thought to arise from sterility, although higher heterozygosity or more genetic material resulting in faster transcription may also play a role (Beaumont and Kelly, 1989). Whatever the method is that infers the advantage, it varies considerably between families and even between species. The main advantage that can be expected from triploidy is an increased growth rate. This has previously been observed in many species, including *Ostrea edulis* (Hawkins, *et al.*, 1994), *Crassostrea gigas* (Hawkins, *et al.*, 2000) and *Mytilus edulis* (Beaumont and Kelly, 1989).

Further advantages that exist through triploidy are exclusively a result of sterility, or reduced gametogenesis as observed in some species of Haliotids (Stepito, 1997; Zhang, *et al.*, 1998; Liu, *et al.*, 2004). The sterility or retarded gonadosomatic activity will result in an improved product yield percentage, biosecurity advantages and improved management options (Beaumont and Kelly, 1989).

Any of the above-mentioned advantages will have economic impacts on shellfish aquaculture and warrants investigation in *H. midae*.

## **2.3 Effects of triploidy on production traits**

The effects of triploidy vary greatly amongst species and some of the advantages include faster growth, improved yield and superior product quality (Schoonbee, 2008).



### 2.3.1 Effects on growth

Various authors have reported differential growth rates between triploid and diploid genotypes in molluscs with triploids displaying a superior growth rate in *Haliotis midae* (Stepto, 1997), *H. discus hannai* (Zhang, *et al.*, 1998) and *H. rubra* (Liu, *et al.*, 2004). There are a number of theories providing an explanation to the superior growth rates observed in triploids.

The “triploid advantage” theory suggests that triploid individuals are sterile and energy used for gametogenesis in diploid genotypes is available to support somatic growth in triploid genotypes (Tabarini, 1984; Allen, *et al.*, 1986; Barber and Mann, 1991; Ruiz-Verdugo, *et al.*, 2000).

Some triploid populations may display heterosis because of an increased heterozygosity. This is because there is a higher probability of having more than two different alleles at each gene. This is also referred to as hybrid vigour (Beaumont and Fairbrother, 1991; Hawkins, *et al.*, 2000; Magoulas, *et al.*, 2000; Garnier-Gere, *et al.*, 2002). Diploids might even show decreased growth compared to triploid genotypes because of higher homozygosity. Higher levels of homozygosity can result in a higher level of expression of deleterious mutations in the diploid state (Zouros, *et al.*, 1996).

The gene-dose hypothesis implies that for a triploid genotype there are three homozygous alleles at each gene therefore three times the gene product might be available. The gene products that affect or increase growth can also be transcribed faster because there are three gene templates available in triploids compared to the two in diploids (Magoulas, *et al.*, 2000).

Triploid genotypes also demonstrates a general increased cell size as the cells have to accommodate more DNA due to the presence of three sets of chromosomes in the nuclei (Guo and Allen, 1994b; Yang, *et al.*, 1998).

Triploid genotypes induced by the retention of the first polar body is referred to as Meiosis I triploids and similarly triploids induced by the retention of the second polar body as Meiosis II triploids (Hawkins, *et al.*, 1994). Differential growth rates have also been reported between Meiosis I and II triploids in *Crassostrea virginica* (Stanley, *et al.*, 1984), *Mytilus edulis* (Beaumont and Kelly, 1989), *Ostrea edulis* (Hawkins, *et al.*, 1994)

and *Crassostrea gigas* (Hawkins, *et al.*, 2000). In all of these species the Meiosis I triploids outperformed the Meiosis II triploid and/or diploid genotypes. It was also shown that the heterozygosity was the highest in Meiosis I triploids, followed by Meiosis II triploids and then by the diploids. The authors concluded that heterosis was the reason for higher growth rates of the triploid genotypes.

Contrary to the above-mentioned results, Garnier-Gere *et al.* (2002) compared Meiosis II triploids to diploids of the species *Crassostrea gigas* in different environments. They found that the mean heterozygosity in the Meiosis II triploids was higher than that of the diploids, but the heterozygosity levels overlapped. The triploids outperformed the diploids in both environments and the faster growth was not as a result of heterosis but rather triploid advantage (Garnier-Gere, *et al.*, 2002).

Triploid abalone of the species *Haliotis discus hannai* (Zhang, *et al.*, 1998), *H. midae* (Stepito, 1997) and *H. rubra* (Liu, *et al.*, 2004) all showed superior growth when compared to diploids in the individual experiments. In similar studies in *H. laevigata* (Elliot, *et al.*, 2004), *H. midae* (Schoonbee, 2008) and *H. rubra* (Liu, *et al.*, 2009) the authors, however, found no differences in growth rates. There is no definite theory or hypothesis that explains the specific cause of differential growth or lack thereof in a triploid genotype of a particular species, neither a way to determine which combination of theories result in the most probable explanation for the observed differences in growth.

### **2.3.2 Effects on yield and quality**

A study on *H. rubra* (Liu, *et al.*, 2009) gave no indication of differential growth rates between diploid and triploid individuals on the basis of shell length and body weight. A significant increase in yield percentage in triploid individuals, compared to diploids, was however reported. The triploid individuals had a more elongated shell and larger foot muscles in comparison to their diploid controls. The triploid individuals did not display gonadal development while their diploid counterparts reached sexual maturity and spawned during the course of the experiment. The triploid abalone could be identified as having either brown-yellow coloured or no gonads when compared to the mauve or

cream-white gonads of the diploids. The triploid individuals also appeared not to have any gametogenic development during the time of the trail (Liu, *et al.*, 2009).

Sterility and the consequent reduced spawning activity will allow live abalone, which is a primary method of export, to be marketed throughout the year without fear of reduced quality due to random spawning during transport (Beaumont and Fairbrother, 1991).

Shell quality is used as an indicator of quality in abalone and this in turn is determined by on-farm management practices such as regular grading. Grading is done to limit size variation and maintain standard stocking densities. Handling is the major cause of breakage or shell damage in production animals. In a trial on triploid clams (*Mya arenaria*) (Mason, *et al.*, 1988), there was a significant reduction in the variance of physiological and morphological parameters due to an increased heterozygosity. These results may indicate the possibility that triploid populations of shellfish can display less variation in growth. This will minimize the need for grading and improve product quality (Schoonbee, 2008).

### **2.3.3 Effects on survival**

In triploid oyster larvae (*Crassostrea gigas*) the initial mortality rate is higher than in diploids. However, in later life stages the mortality rates were equal for triploid and diploid individuals. In this same study the survival rate of diploids were higher than that of triploids during settlement, but equal after that (Garnier-Gere, *et al.*, 2002).

Later in life, disease resistance becomes more important and reports indicate the probability that triploids have improved disease resistance as well as improved resistance to other stress factors (Allen and Downing, 1986; Hawkins, *et al.*, 2000). This is probably related to the fact that sterility and increased heterozygosity in triploids resulted in a lower requirement for metabolic energy leaving more energy to support the immune system during stressful conditions (Hawkins, *et al.*, 2000; Magoulas, *et al.*, 2000).

In the study on *Haliotis rubra* (Liu, *et al.*, 2009) the mortality rate over a 30-month period showed no apparent variation between triploid and diploid genotypes. A higher mortality

rate is associated with the induction of triploidy, but the triploid state *per se* has not been reported to negatively affect the survival of molluscs.

## 2.4 The use of triploidy for biological containment

A need exist for the biological containment of fish and shellfish from commercial farming activities to avoid genetic contamination of surrounding wild populations. The sterility associated with triploidy may be exploited to ensure biological containment. This containment may be required to constrain the excessive multiplication of highly prolific species or act as a safeguard against the threats of competition or predation imposed on natural populations by escapees of exotic species.

Containment of domestic stock has become more important recently due to the expansion of aquaculture activities into environments were these species are not native or indigenous. The reproductive sterility associated with triploidy presents such a method of containment. The main restriction is however that triploid induction is not 100% effective (Piferrer, *et al.*, 2009) and reversion of as high as 20% to heteroploid mosaics or diploids remains inherent (Allen and Guo, 1996).

In trials on triploid individuals, the so-called sterile triploids developed gonads and even spawned in some instances. These trials include a study by Allen and Guo (1996) where they found mosaics in their triploid stocks of *Crassostrea gigas*. These results confirmed that triploidy is unstable, but that the mosaics became sexually active could not be proved. Triploid Pacific oysters (*Crassostrea gigas*) that showed retarded and abnormal gonadal development were still capable of spawning. It was attributed to an environmental response to sperm in the water (Allen and Downing, 1986). In a similar trial, Guo and Allen (1994a) found that such gametes produced by triploid Pacific oysters were capable of fertilization but resulted in aneuploid progeny, which did not survive.

In triploid *Haliotis laevis* the females did not develop gonads and the males showed some gonad development by the age of four years. Both were unable to spawn during conditioning or when receiving spawning cues (Dunstan, *et al.*, 2007). Liu, *et al.* (2009)

suggested that *H. rubra* triploids showed severely retarded gametogenesis and that it was highly likely that offspring of these triploids would not be viable.

Triploidy on its own does not prevent reproductive activities. Gametogenesis, gamete maturation and spawning are all under control of sex steroids and hormones and environmental signals (Liu, *et al.*, 2009). Although there is evidence that triploids are not 100% sterile on the basis of gametogenesis or gonadal maturation, in most instances thus far successful reproduction has remained virtually zero.

Triploidy is an appropriate method to reduce or eliminate the genetic impact on the natural population. If this is the chosen method to induce sterility, a precautionary two full consecutive reproductive cycles should be monitored to confirm functional sterility (Piferrer, *et al.*, 2009).

## **2.5 Methods for induction of triploidy**

The efficiency of any treatment used to induce triploidy is dependent on three main parameters (Schoonbee, 2008).

- 1.) The treatment conditions (cold or heat in thermal shock, pressure intensity in hydrostatic pressure shock, kind and concentration of chemicals in chemical shock).
- 2.) The duration of the shock treatment.
- 3.) The timing of the treatment in terms of the cell or meiotic cycle.

### **2.5.1 Chemical treatment**

The most commonly used chemical to induce triploidy is Cytochalasin B, referred to as the CB treatment. CB is a fungal metabolite that is thought to inhibit actin polymerization, thereby inhibiting micro-filament formation in cells (Stepito and Cook, 1998).

This method of induction requires less specific duration of treatment and produces higher percentages of triploidy than other treatments. The chemical is expensive and induces high levels of mortalities during larval development (Stepito, 1997). This is

because CB tends to produce higher levels of ploidy than required, resulting in polyspermy and abnormalities (Beaumont and Fairbrother, 1991; Gérard, *et al.*, 1999).

CB treatment is administered by dissolving it in dimethylsulphoxide (DMSO) before preparing at a specific concentration of 0.5 to 1.0mg/l in natural seawater. Fertilized eggs are placed in this solution for the required 15 minute period and then washed in a solution of DMSO and seawater to remove the remaining CB. Treated fertilized eggs are then incubated in normal seawater (Beaumont and Fairbrother, 1991).

Other chemicals used to inhibit polar body release, thereby inducing triploidy, are 6-dimethylaminopurine (6-DMAP), calcium and caffeine. Caffeine and calcium resulted in poor larval development and inefficient triploid induction. 6-DMAP is relatively safe to use with relatively high levels (64%) of success reported in *H. asinina* (Norris and Preston, 2003; Scarpa, *et al.*, 1994).

### **2.5.2 Thermal treatment**

Thermal treatment is a physical method of triploid induction whereby the temperature shock interferes with normal meiosis and mitosis (Beaumont and Fairbrother, 1991) and once the temperature is normalized, normal embryonic development resumes. It is thought that the temperature change prevents polar body extrusion by changing the various development rates and thereby interfering with the meiotic microtubules or by changing the density in the cytoplasm (Piferrer, *et al.*, 2009).

Temperature shocks are more variable than other physical treatments and can be either heat or cold treatments. Temperatures for heat shock range from 25-38°C and cold shock from 0-5°C. It is however, the difference between the normal functional temperature of the particular species and the temperature treatment that is more important in this induction method (Beaumont and Fairbrother, 1991; Piferrer, *et al.*, 2009).

Cold shocks are suitable to use with fish that have small eggs but fish with large eggs show higher intrinsic variations to inductions using temperature shock (Piferrer, *et al.*, 2009). This method has not been widely used in molluscs but recent research showed

that thermal treatment on *H. diversicolor* (Yang *et al.*, 1998), *H. discus hannai* (Arai *et al.*, 1986) and *H. midae* (Stepito, 1997) resulted in high proportions of triploids (Schoonbee, 2008). This is very useful in regions including the European Union that does not allow the induction of triploids using cytochalasin B (Piferrer, *et al.*, 2009).

### **2.5.3 Pressure treatment**

Timing and intensity are the two most important variables to consider when it comes to any physical induction treatment. Although hydrostatic pressure as a method of triploidy induction has been reported in shellfish, it is more commonly used in finfish species (Piferrer, *et al.*, 2009). Initial results indicate that the physical induction methods, temperature and pressure, were less successful than the chemical treatments, yielding lower percentages of triploidy at higher rates of mortality (Beaumont and Fairbrother, 1991).

Pressure treatments involve the application of brief and sudden increases in hydrostatic pressure that is applied to fertilized eggs. The mechanism has not been determined but probably acts directly on the oolemma preventing the extrusion of the polar body or affects the meiotic spindle (Piferrer, *et al.*, 2009). The limitation of this treatment is that it arrests all development and only the eggs that are at the susceptible stage of division will be affected by the treatment. This also applies to thermal treatments (Beaumont and Fairbrother, 1991; Griffiths, 1994).

Pressure shock treatment also requires a special pressure vessel that can handle very high pressures (Beaumont and Fairbrother, 1991). Pressure treatment to induce triploidy has been more successful in finfish species than in shellfish whilst relatively low triploid levels have been achieved in molluscs (De Beer, 2004). De Beer (2004) applied hydrostatic pressure to *Haliotis midae* and was the first to report high levels of triploidy, in the range of 95-100%.

#### **2.5.4 Use of tetraploids in triploid production**

The use of chemical and physical inductions does not produce 100% triploidy and usually results in some mortality. With the use of tetraploid brood stock that is crossed with diploids these restrictions can be overcome (Allen and Guo, 1996).

Guo *et al.* (1996) suggested that using tetraploid crosses is better suited to produce triploid progeny in aquaculture than altering meiosis. Lutz (2001) also recommended the use of tetraploid parents to produce triploids rather than the chemical or physical treatments. The mating of tetraploids with diploids to produce triploid progeny has become more common and has been increasing but the only way to accomplish this is through viable tetraploid parents that produce stable diploid gametes that can be fertilized (De Beer, 2004; Piferrer, *et al.*, 2009).

The only successful method of producing tetraploid brood stock is by blocking polar body I in viable eggs of triploids and fertilizing it with sperm from diploids (Guo and Allen, 1994a). Physical and chemical inductions should however, not be abandoned since it is required to induce triploid brood stock in species where tetraploids are not available (Schoonbee, 2008).

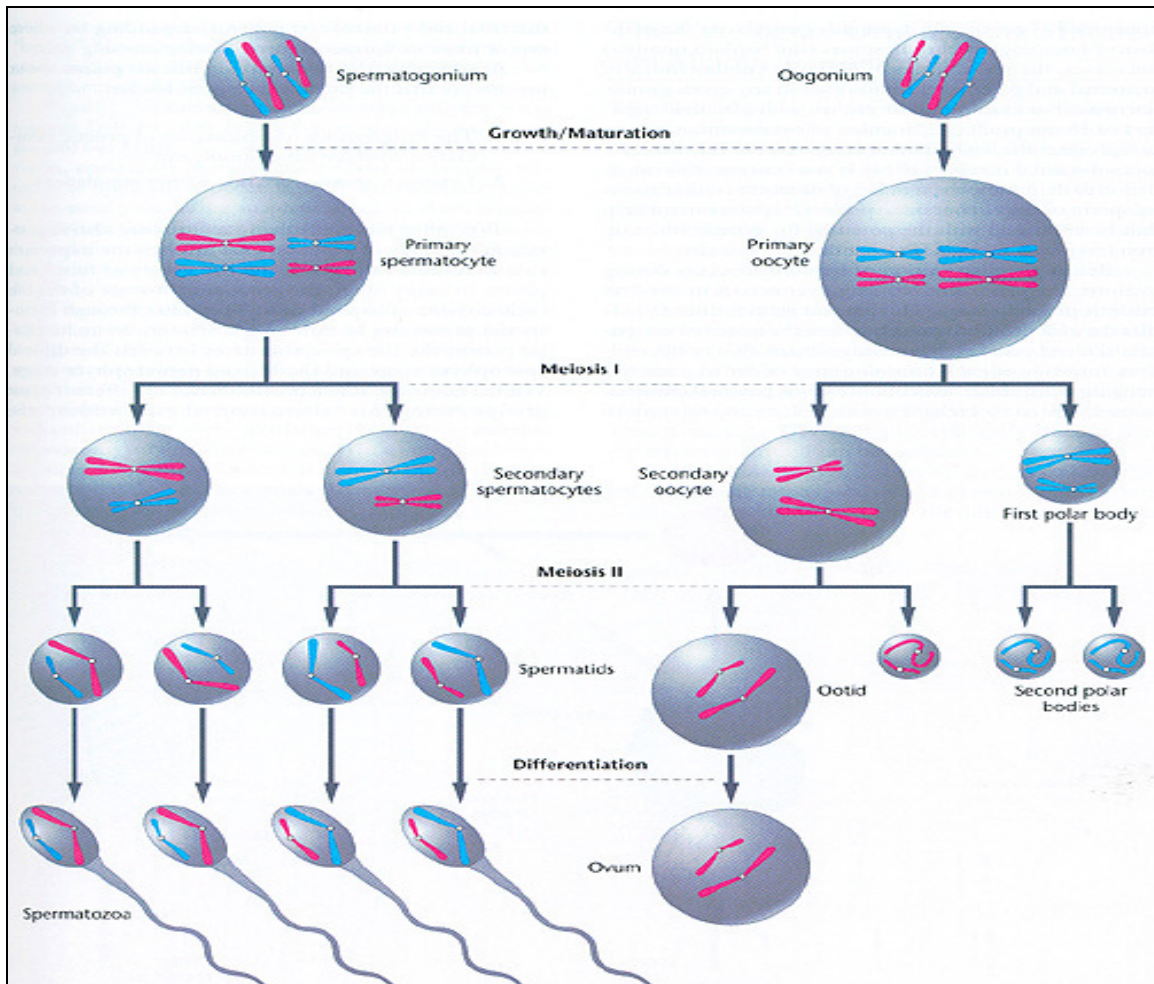
Another aspect to consider when using tetraploids is the environmental risk associated with the escapee or accidental release of reproductive tetraploids into the wild. For this reason, correct measures, such as quarantine, must be taken to ensure that tetraploid brood stock used for commercial or experimental purposes do not pose a genetic threat to the environment (Piferrer, *et al.*, 2009).

## **2.6 Induction of triploidy by polar body retention**

Before germ cells become gametes, they go through two maturation divisions in Meiosis I and Meiosis II (Figure 2.3). Meiosis begins with DNA replication and synapsis of chromosome pairs. At the end of Meiosis I, the chromosome pairs segregate and one chromosome from each homologous pair goes to a daughter cell. This halves the chromosome number.



In Meiosis II, there is a division of each chromosome into two daughter chromatids. These sister chromatids from one chromosome split and goes to opposite poles of the nucleus. A nuclear envelope forms around each of the chromosomes at the two poles and the cell divides to form new cells called gametes. These gametes have a haploid chromosome number (Beaumont and Fairbrother, 1991).



**Figure 2-3** Gametogenesis and the formation of the two polar bodies (image available at: <http://legacy.owensboro.kctcs.edu/gcaplan/bio/Notes/BIO%20Notes%20D%20Cell%20Division%20&%20Karyotypes.htm>)

Male meiosis produces four gametes (sperm cells) while female meiosis only produces one gamete (ovum). This dissimilarity in gamete number between male and female occurs because female germ cells have unequal division of cytoplasm. In Meiosis I after the division, there are two cells with equal chromosome constitution but one with almost all the cytoplasm. The cell with all the cytoplasm is called the secondary oocyte and the

other cell the first polar body. In Meiosis II, the secondary oocyte undergoes division and forms the ovum or egg and another polar body. This is called the second polar body.

In normal reproduction and fertilization, neither of the two polar bodies contributes any chromosomes to the final zygote. The polar bodies are eventually degraded and the chromosomes they contained, lost (Fairbanks and Anderson, 1999).

### **2.6.1 Meiosis I triploidy compared to Meiosis II triploidy**

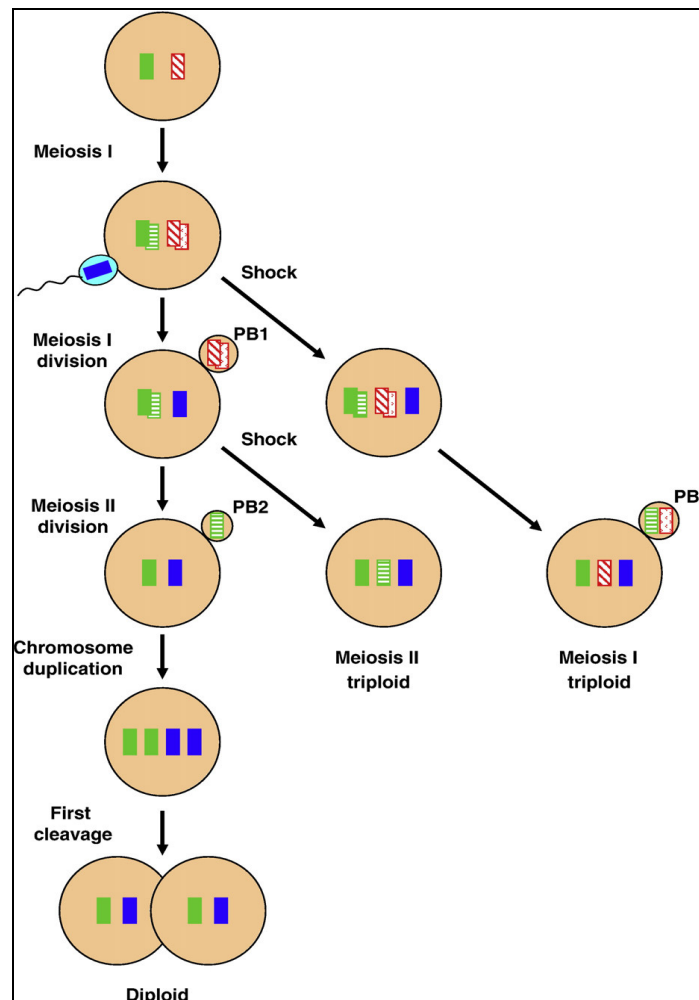
In a study of *Ostrea edulis* triploidy (Hawkins, *et al.*, 1994), Meiosis I triploids outgrew Meiosis II triploids and diploids by 60%. They found that the average multiple locus heterozygosity was 50.5% higher in the Meiosis I triploid siblings, compared to the other two groups, accounting for the faster growth rate. This corresponds with results in similar experiments on *Crassostrea gigas* (Hawkins, *et al.*, 2000) and *Mytilus edulis* (Beaumont and Kelly, 1989) where allelic variation (measured as multi-locus enzyme heterozygosity) was the highest in Meiosis I triploids, but Meiosis II triploids higher than diploids.

Allelic variation results in high heterozygosity, which affects physiological performance, and may account for the faster growth rate in Meiosis I triploids. Stanley *et al.* (1984) observed faster growth in *Crassostrea virginica* Meiosis I triploids. These outgrew the diploids and Meiosis II triploids and the authors concluded that the faster growth was a result of higher heterozygosity, which was observed in the Meiosis I triploids, rather than triploid advantage.

Garnier-Gere *et al.* (2002) concluded that there was a triploid advantage in spite of heterozygosity and that triploid advantage could exist in both favourable and unfavourable environments. They compared diploid and Meiosis II triploid *Crassostrea gigas* in different environments. Meiosis II triploids grew faster in both environments and their average heterozygosity was higher than diploids, but the ranges of both overlapped considerably. This indicated triploid advantage regardless of heterozygosity level.

Contrary to these results above, there was no difference in growth rate between Meiosis I and Meiosis II triploids of the Pacific abalone (*Haliotis discus hanna*) and Meiosis I triploids had higher mortalities and abnormalities than Meiosis II triploids (Zhang, *et al.*, 1998). Meiosis II triploids of the South African abalone, *H. midae*, also showed a higher survival rate when compared to Meiosis I triploids (Stepito, 1997).

In a study to compare the induction methods for Meiosis I and II triploids, Norris and Preston (2003) concluded that the blocking of polar body II would be the favoured method used in commercial scale triploid abalone production. This resulted from a much higher survival rate in Meiosis II triploids and the physical ease to block the second polar body. Figure 2.4 shows how triploidy is induced by blocking the polar bodies.



**Figure 2-4** Triploid induction by retention of the first or second polar bodies (Piferrer, *et al.*, 2009)

From the above it becomes apparent that the exact reasons for triploid advantage have not been established yet, but only that there is evidence of advantage and the accurate quantification of this advantage is compounded by the fact that different species react differently to the induction (Schoonbee, 2008).

## 2.7 Verification of triploidy

Any induction of ploidy, such as triploidy, need to be validated in terms of the actual level of ploidy achieved in the targeted species. Methods to verify the level of ploidy exists and is well developed. Such methods may be direct and involve karyotyping, the measurement of DNA content, genotyping of microsatellite DNA markers or nuclear organising region (NOR) analysis. There are also indirect methods including nuclear sizing or cell size measurements. Any polyploidy results in a higher number of chromosomes and results in a larger volume of DNA in the nucleus (Piferrer, *et al.*, 2009). The nuclei and actual cells of the triploids are generally larger (triploid gigantism) and can be compared to the size of diploids (Ihssen, *et al.*, 1990).

The level of ploidy achieved through induction requires a reliable method that can precisely determine the level of ploidy and that can be used in the verification of large numbers of individuals in an affordable manner within a reasonable timeframe. One such method that has been widely used in the animal production industry is flow cytometry (Allen, 1983; Lecommandeur, *et al.*, 1994) with microsatellite markers recently developed offering another verification method (Slabbert, *et al.*, 2010).

## 2.8 Summary

Induction of triploidy is currently widely practised with regard to commercial production of various shellfish species, including *Crassostrea virginica* (Utting, 1995), *C. gigas* (Allen and Guo, 1996), as well as other oyster species (Garnier-Gere, *et al.*, 2002; Davis, 2004). Other species where triploidy is used in commercial applications are *Tapes philippinarum* (Liang and Utting, 1994), *Mytilus edulis* (Brake, *et al.*, 2004), *Argopecten irradians* (Tabarini, 1984) and *Chlamys farreri* (Yang, *et al.*, 2000).

The advantages arise from sterility or retarded gonadosomatic development, and may include superior growth, improved product quality and increased yield, but the effects are varied and in most instances, no clear explanation for the associated advantage/s exists (Piferrer, *et al.*, 2009).

Triploidy in abalone is not well established and results are conflicting. In some Haliotids there is a clear growth advantage; *Haliotis midae* (Stepito, 1997), *H. discus hannai* (Zhang, *et al.*, 1998) and *H. rubra* (Liu, *et al.*, 2004). Other studies on the same species have however shown no growth advantage. The induction method may only result in partial triploidy or triploid mosaics, and depending on the cells affected, differential results may be obtained. Superior performance in abalone is dependent on many environmental factors, hormonal concentrations and managerial influences. Many parameters interact to determine the final result.

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### **3. Verification of the ploidy status of experimental material used in the comparative growth trials of abalone, *Haliotis midae***

#### **3.1 Introduction**

This experiment is a continuation of the work initiated by Schoonbee (2008) on the comparative growth of triploid and diploid variants of the South African abalone *Haliotis midae*. Schoonbee (2008) compared the growth (measured as wet weight and shell length) of triploid and diploid siblings from age eight to 24 months. No significant difference in growth was detected during this growth phase (Phase 1) and a recommendation was made that the trial be continued beyond the age at which sexual maturity is reached, when the advantages associated with triploidy such as sterility and superior growth could come in to effect.

In the wild, the South African abalone matures only after three to five years, though this is correlated to size. Abalone grows faster under farmed conditions and may reach the reproductive size of 80 to 100mm shell length at an age of 30 to 36 months (Sales and Britz, 2001). This implies that gonadal maturation will occur in the growth phase beyond 24 months of age during which the effect of triploidy and its potential advantages stand a better chance to manifest.

Schoonbee (2008) tagged all individuals according to the treatment method, i.e. triploid or diploid. She confirmed the ploidy status of material on two occasions during the experimental period. The first analysis was performed two days after fertilization and induction through the method of flow cytometry. The results confirmed triploid percentages of 95-100% amongst repeats within the triploid treatment group (De Beer; 2004; Schoonbee, 2008). A second validation of triploidy was conducted at the end of the Phase 1 growth trial, at age 26 months, also with the use of flow cytometry. Fifteen individuals were randomly sampled from the treatment group and the results confirmed a triploidy level of 93% was maintained with only one individual showing a loss of aneuploidy (Schoonbee, 2008).

Verification of the ploidy status of experimental material is an essential component in the comparison of growth between triploid and diploid genotypes, and the ability to

confirm that reversion (a loss of the aneuploid state) from triploid to diploid has not occurred. It is therefore important to have a method to determine the level of ploidy of a large number of individuals, in an affordable manner, within a reasonable time.

This Chapter presents the methods used to validate the ploidy status during the second growth phase (Phase 2) from age 29 to 62 months and again towards the end of the trial. This was done to confirm that growth comparisons were conducted on the basis of differential ploidy of the treatment groups. Two methods namely flow cytometry and the use of molecular markers specifically developed for *Haliotis midae*, will be presented and compared in terms of their suitability for the verification of ploidy in commercial abalone farming.

### **3.1.1 Flow Cytometry**

Flow cytometry is based on the principles of light scattering, light excitation and the emission of fluorochrome molecules and is therefore the measurement of the physical or chemical characteristics of cells. As cells intercept the light source they scatter the light and this excites the fluorochromes into a higher energy state. The energy is released as a photon with specific spectral properties. These are unique for different fluorochromes (Ormerod, 1999).

The cells or particles move through the measuring point in a fluid stream that enables the measurement of thousands of cells or particles within a short period of time. The data generated by the fluorescence and scattering of light is processed by a computer program into histograms for analysis (Shapiro, 1994; Ormerod, 1999).

Mack Fulwyler built one of the first flow cytometers in the early 1960's at Los Alamos but since then it has been a rapidly growing technology. Most of the modern instruments use a low power, air-cooled argon ion laser with a fixed emission light wavelength at 488 nanometers. Currently, the most important application of flow cytometry is the clinical application for immunological characterization of lymphomas and leukaemias, cross matching of organ transplant tissues and counting lymphocyte subpopulations in peripheral blood of HIV-infected patients (Mandy *et al.*, 1995).

### 3.1.1.1 Application of flow cytometry

Flow cytometry is versatile in that a number of cell properties can be measured, including the DNA content of nuclei, the expression of a surface antigen, the activity of intracellular enzymes, the RNA or protein content and chromosome analysis (Vindeløv and Christensen, 1994; Ormerod, 1999).

In clinical tumour sample analysis the DNA content of a cell can give an indication of the proliferation of the tumour. The fraction of cells that are in the S phase (synthesis phase) can indicate whether there is active growth or division in the tumour. Dormant cells, which are not actively growing or dividing, are referred to as being in the  $G_0$  state. As cells prepare to divide, it enters the  $G_1$  phase and proteins necessary for DNA replication are synthesized. Most somatic cells are arrested during the  $G_1$  phase. The S phase follows the  $G_1$  phase during which the DNA content increases until it has doubled. DNA synthesis ceases and the cell enters the  $G_2$  phase. The cell cycle continues from the  $G_2$  phase to mitosis, which results in the formation of two daughter cells (division of the DNA in  $G_2$ ) (Fairbanks and Anderson, 1999; Ormerod, 2000).

Similar to the analysis of tumour samples in clinical flow cytometry, the ploidy of cells can also be measured. The DNA content can indicate the state of ploidy that the cell is in. This is under the assumption that level of fluorescence is proportional to ploidy (Griffiths, 1994). The use of flow cytometry for the assessment of ploidy in shellfish has become the most popular method since it was first reported by Allen (1983) on triploid oysters and clams. Flow cytometry is a fast and accurate procedure that can be applied non-destructively (animals are kept alive) on many tissue types including hemolymph, gill, mantle, foot tissue, larvae and spat (Allen, 1983; Chaiton and Allen, 1985; Yang, *et al.*, 2000).

In this application flow cytometry measures the fluorescence of the cell nucleus after it has been stained with a DNA intercalating fluorescent dye. These dyes include propidium iodide (PI), Ethidium Bromide, Acridine Orange or 4',6-diamidino-2-phenylindole (DAPI). The amount of dye absorbed is proportional to the amount of DNA in the cell nucleus. Triploid cells will have stained nuclei that emit one and a half times

the fluorescence of diploid nuclei (Allen, 1983; Chaiton and Allen, 1985; Nell, 2002; Liu, *et al.*, 2004).

Flow cytometry for ploidy analysis has been used comprehensively on triploid induced Haliotids such as the Australian blacklip abalone, *Haliotis rubra* (Liu, *et al.*, 2004; Liu, *et al.*, 2009), 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* (Stepito, 1997; De Beer, 2004) as well as various oyster species (*Crassostrea gigas* and *Ostrea edulis*) (Allen, *et al.*, 1986; Supan, *et al.*, 2000).

### 3.1.2 Molecular markers

There are too many aquaculture species that require the development of molecular markers to simply sequence their entire genomes (Liu and Cordes, 2004). Instead other approaches are needed to develop genetic markers. These include dominant amplified fragment length polymorphism (AFLP) markers (Vos, *et al.*, 1995) used where genome sequencing or QTL mapping resources are not available (Martinez, 2007); expressed sequence tags (ESTs) from complimentary DNA (cDNA) libraries or sequenced genes (O'Brien, 1991; Panitz, *et al.*, 2002; Hayes, *et al.*, 2004; Rise, *et al.*, 2004) and microsatellite markers.

Microsatellite markers are simple sequence repeats (SSRs) arranged in tandem arrays in the genome (Martinez, 2007). They are a favoured marker because they show co-dominant expression, Mendelian inheritance, high polymorphic information and are abundant in the whole genome (Wright and Bentzen, 1994). There are two types of microsatellite markers. The first is markers, which are linked to genes of known function, and the second is markers, which are not linked, also referred to as Type I and II markers, respectively (O'Brien, 1991).

Microsatellite markers can be developed using either genomic libraries or from known gene sequences or EST databases (Martinez, 2007). The first method uses a genomic library to develop clones that bear specific short sequence repeats (SSRs). These clones are then sequenced and used to identify microsatellite-bearing sequences and primers are developed to amplify the regions containing specific SSRs. Individuals from



the target population are then screened to identify any duplicate loci or null (non amplifying) alleles (Coulibaly, *et al.*, 2005). The second method using known genes or ESTs to generate microsatellite markers has been used in many fish species including carp (*Cyprinus carpio*) (Yue, *et al.*, 2004), rainbow trout (*Oncorhynchus mykiss*) (Rexroad and Palti, 2003; Coulibaly, *et al.*, 2005) and Atlantic salmon (*Salmo salar*) (Ng, *et al.*, 2005; Vasemagi, *et al.*, 2005).

Microsatellite markers can be used in a number of applications to aid in the conventional breeding of aquaculture species, as well as a tool for the verification of triploidy, as in the case of the abalone *Haliotis midae* that will be reported on in this study. In induction of triploidy in abalone, one of the polar bodies are retained resulting in two sets of chromosomes from the female parent and one set from the male parent (Gérard, *et al.*, 1999). If there is a heterozygous locus in the female and the male inherited allele has a different size than the two female inherited alleles, microsatellite markers become useful (Slabbert, *et al.*, 2010b).

Many loci need to be screened to identify a combination of markers that can reliably identify triploid individuals and this became possible for *Haliotis midae* with the development of more than 200 microsatellite markers (Bester, *et al.*, 2004, Slabbert, *et al.*, 2008; Slabbert, *et al.*, 2010a).

Thirty polymorphic microsatellite loci were tested in control groups and a final microsatellite multiplex consisting of seven markers was optimized. Forty-two abalone with unknown ploidy were successfully identified as either triploid or diploid using the microsatellite multiplex protocol developed by Slabbert *et al.* (2010b), confirming the use of identified microsatellite markers to verify the ploidy level of *Haliotis midae*.

## **3.2 Materials and Methods**

### **3.2.1 Material**

In 2006 three distinct progeny groups (Group A, B and C) were produced by consecutive spawning events three to four months apart (Schoonbee, 2008), summarized in Table 3.1. Each spawning event utilized six individuals randomly

selected from a group of 15 sexually mature females and 10 males. The males and females were induced to mass spawn using standard hatchery procedures as used by Liu, *et al.* (2004). The eggs were collected by siphoning them from the bottom fertilization trays and 200 millilitres (mL) of concentrated eggs, filtered through a 400  $\mu\text{m}$  sieve, immersed in 17 °C seawater was then fertilized with 25 mL sperm using standard fertilization procedures. Three to four fertilizations and triploid inductions had to be performed during each spawning event due to the limited volumetric capacity of the pressure induction equipment of 150 mL. The broodstock were induced to spawn and equal volumes were used during each fertilization ensuring similar contributions from each parent (Schoonbee, 2008).

After fertilization, the zygotes were collected and placed into the pressure vessel. It consists of a stainless steel cylinder with a volume of 150 mL and a headpiece, that seals with a rubber o-ring, with a pressure gauge and valve. The cylinder fits into a steel frame that allows the cylinder and headpiece to be clamped between the top of the frame and a hydraulic jack at the bottom as seen in Figure 1.1. Pressure was applied to the zygotes according to the recommendations by De Beer (2004).

A random sample of larvae was taken 48 hours after fertilization, analyzed using flow cytometry and a near 100% triploid state was reported. The animals were thereafter maintained according to a procedure described by Schoonbee (2008) and tagged at the age of eight months old (with the smallest individuals 5mm long). Both diploid and triploid genotypes were then entered into a growth trial with measurements taken every month to the age of 24 months when growth results were reported (Schoonbee, 2008). The ploidy status of the animals was verified by Schoonbee (2008) at 26 months of age at which they displayed 93% aneuploidy and were expected to be functionally sterile.

For this experiment the original three Groups produced by Schoonbee (2008), consisting of triploid and diploid siblings, was used to conduct a second comparative growth phase from the age of 29 to 62 months. Schoonbee (2008) reported no significant difference in growth between these Groups and they were therefore considered as Repeats during the Phase 2 growth period. Given the possibility of reversion from triploid (Allen and Guo, 1996) to the diploid state during the second

growth phase, it was considered important to verify the triploidy status at the end of the second growth trial (month 62).

**Table 3-1** The three treatment groups generated by Schoonbee (2008) used during Growth Phase 1 and 2, on which triploidy verification were conducted at age 48 hours and 26 months

Group	Spawning Date	Treatment		Triploid Verification	
				48 hours	26 months
A	March 2004	Standard	Induced	100%	93%
B	June 2004	Standard	Induced	100%	Not sampled
C	October 2004	Standard	Induced	100%	Not sampled

Standard = Standard hatchery procedures as described by Liu *et al.* (2004).  
 Induced = Triploidy induction as described by De Beer (2004).

In addition to flow cytometry as the preferred method to verify ploidy (Schoonbee, 2008), a series of more than 200 microsatellite markers has been developed for *Haliotis midae* by the molecular research unit at the Department of Genetics at Stellenbosch University (Bester, *et al.*, 2004; Slabbert, *et al.*, 2008; Slabbert, *et al.*, 2010a). From these markers, 30 polymorphic microsatellite loci was investigated for application in the development of a high-throughput protocol for the verification of triploidy (Slabbert, *et al.*, 2010b). Both methods were then used to verify the ploidy status of individuals at the end of the second growth period (month 62).

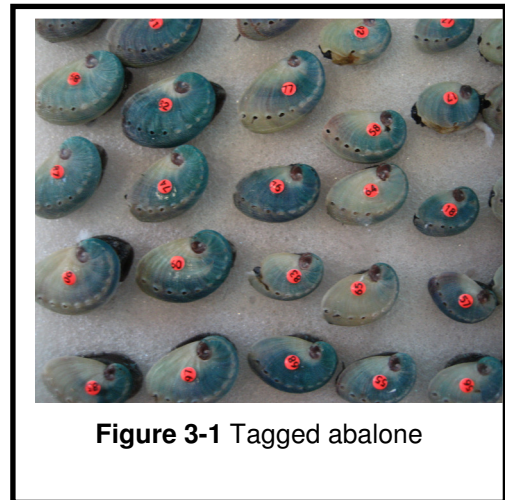
## 3.2.2 Methods

### 3.2.2.1 Sampling

During Phase 1 Schoonbee (2008) measured the growth of three groups up to the age of 24 months. By the time that Phase 1 ended, the number of animals that could be sampled had decreased due to tag loss. Once the triploid or diploid individual had lost its tag, it became an unknown individual and could not be included in the trial. The

abalone were graded every six months to ensure correct stocking densities according to standard farm practices. Every time a basket within a group was graded, it became a Repeat in the trial. The number of tagged individuals within each Repeat at the beginning of Phase 2 was low and it was necessary to increase the amount by retagging. In order to retag an individual it had to be verified as either triploid or diploid. Initial samples were taken at 46, 49 and 52 months of age during Phase 2.

During each sampling event, 99 unknown individuals (tag lost) were randomly sampled and marked with a plastic bee tag (Figure 3.1). This allows the sample to be verified as either triploid or diploid and the individual can again be entered into the trial. In the beginning of the experiment, Schoonbee (2008) entered 300 triploid and 300 diploid animals into each Group. Assuming random mortalities and tag loss, the 99 unknown individuals should show a 50 to 50 ratio of diploid and triploid individuals.



**Figure 3-1** Tagged abalone

The experimental design and number of individuals entered into each method of verification can be seen in Table 3.2. The diploid individuals were used as a control for each method. Only eight triploid and eight diploid samples from the 52-month old sampling was used to optimize the microsatellite multiplex. After this initial verification and the optimization of the microsatellite multiplex, every time that a canning trial was performed six triploid individuals were randomly sampled and verified using both methods in direct comparison to determine the current ploidy state.

All animals were anesthetized by standard commercial practices using either  $MgSO_4$  or  $CO_2$ . Animals were then randomly selected according to the number presented in Table 3.2 and a tissue sample was taken by removing an epipodium and processed in accordance with the method prescribed by Yang, *et al.* (2000). Epipodial tissue was used as a substitute for gill tissue since non-destructive sampling was required.

**Table 3-2** The number of individuals used to calibrate and assess the ploidy level using the two verification methods as well as the unknown and canning trial individuals entered to verify triploidy during Phase 2. It is important to note that the six samples used during the canning trials were all triploid.

Group	Age at sampling	Treatment	Ploidy Verification	
			Molecular Markers	Flow Cytometry
A	52 months	Triploid	8	12
		Diploid	8	6
		Unknown	88	11
	62 months	Canning 1	6 (triploid)	
	66 months	Canning 2	6 (triploid)	
B	49 months	Triploid	0	12
		Diploid	0	6
		Unknown	85	14
	59 months	Canning 1	6 (triploid)	
	63 months	Canning 2	6 (triploid)	
C	46 months	Triploid	0	12
		Diploid	0	6
		Unknown	63	36
	56 months	Canning 1	6 (triploid)	
	60 months	Canning 2	6 (triploid)	

The epipodia samples was cut using sterile equipment and washed in 0.075 M KCl for 10 minutes. After this the samples were placed in Eppendorf tubes filled with either marine phosphate-buffered saline (mPBS) and 10% (v/v) DMSO and frozen at -20 °C for flow cytometry or 99% (v/v) ethanol and stored at room temperature for molecular marker analysis. These samples were analysed using either flow cytometry or the microsatellite markers.

### 3.2.2.2 Nuclear isolation

#### Flow Cytometry

Initially the samples for flow cytometry were processed in a similar method used by Schoonbee (2008). It requires samples to be washed in mPBS and transferred to PI staining solution with 10% (v/v) DMSO (mPBS + 0.1% Triton X100 + 0.2mg/mL RNase A + 0.02 mg/mL PI). The samples were then frozen at -80°C and thawed, followed by vortexing. The fluid was then filtered through a 25 micron nytex screen. After this treatment, samples could be processed by flow cytometry.

However, this method proved problematic in this study and no clear usable results could be obtained. After deliberation with the flow cytometry technician (Ben Loos, October 2009 at Mike de Vries building, Merrimen road, Stellenbosch, 7600) it was decided that the PI staining solution was not a strong enough detergent to dissolve the membrane lipids of the more robust epipodia tissues. It was then decided to use a different method for nuclear isolation.

The CycleTEST™ PLUS DNA Reagent Kit provides a set of reagents for isolating and staining cell nuclei from solid tissue specimens. It involves dissolving the cell membrane lipids with a non-ionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting the cellular RNA with an enzyme, and stabilizing the nuclear chromatin with spermine.

The tissue samples were grinded between two microscope slides and washed into an Eppendorf tube using the provided buffer solution (sodium citrate, sucrose and dimethyl sulfoxide). The cell suspension was centrifuged at 400 times gravity for five minutes. The supernatant was then carefully decanted and 250 µL of Solution A (trypsin buffer) added to the pellet. The mixture was left at room temperature for 10 minutes. After this reaction, 200 µL of Solution B (trypsin inhibitor and RNase buffer) was added and left for another 10 minutes at room temperature. Finally 200 µL of cold (2-8°C) Solution C (propidium iodide staining solution) was added to each tube and left to incubate for 10 minutes in the dark at 2-8°C.

The solution was then filtered through a 25 micron mesh into a suitable container for flow cytometric analysis.

## **Molecular Markers**

For molecular marker verification, the nuclear isolation utilized a different method. All epipodial tissue samples were collected and stored in 100% (v/v) ethanol. During the initial genotyping of eight triploid and eight diploid samples of *Haliotis midae* tentacles, the DNA extractions were performed using the CTAB (N-cetyl-N, N, N-trimethyl ammonium bromide) extraction method. The rest of the DNA extractions were performed using the KAPA Quick Extract kit (KAPA Biosystems) (Slabbert *et al.*, 2010b).

The KAPA Quick Extract is a thermostable protease and buffer that allows rapid extraction in ten minutes inside a single tube, without the need for multiple washing steps. The lysis is performed in a standard thermocycler and the DNA-containing supernatant can be used directly in the polymerase chain reaction (PCR).

The tentacle sample was added to 10  $\mu$ L of the buffer solution, 2  $\mu$ L of the extraction enzyme and PCR grade water. The mixture was incubated for 10 minutes at 60°C in the thermocycler for lysis. The protease was inactivated by incubating the mixture for five minutes at 95°C. Finally, the mixture was vortexed for three seconds and centrifuged at high speed for one minute. The supernatant contained the DNA and 1  $\mu$ L of extract used directly in a 25  $\mu$ L PCR. The PCR multiplex was performed as described by Slabbert, *et al.* (2010a).

### **3.2.3 Results and Discussion**

The purpose of this chapter was to reconfirm triploid status among the animals used in the study, identifying them as aneuploid, thus providing confidence to make conclusions regarding differential results, if any, between the so-called triploid and diploid individuals.

A secondary objective of this specific chapter was to compare and assess the newly developed method of molecular marker analysis to results obtained from the more familiar flow cytometry.

### 3.2.3.1 Data analysis

Initially 36 triploid marked and 18 diploid marked individuals were sampled to reconfirm triploidy using flow cytometry and 46 animals (38 triploid and 8 diploid all from group A) for the development and optimization of microsatellite markers. After this, 236 unknown samples (88 for 52 months, 85 for 49 months and 63 for 46 months) were analysed using microsatellite markers and 61 unknown samples (11 for 52 months, 14 for 49 months and 36 for 46 months) were analyzed using flow cytometry after both methods were calibrated and optimized. Finally, 36 individuals sampled from the two canning trials were verified using both methods. The data was then analysed to:

- a. Confirm aneuploidy in triploid-marked trial individuals.
- b. Determine the ploidy state of genotype unknown individuals by means of flow cytometry and microsatellite markers.
- c. Compare two methods of ploidy verification by assessing flow cytometry and molecular markers for suitability in application to *Haliotis midae* triploidy.

#### a. Confirmation of aneuploidy in trial individuals

In any experimental trial, it is important to have confidence that individuals measured have indeed undergone the treatment. In triploid analysis, the problem is confounded by the possibility of reversion to the diploid state and it becomes necessary to evaluate the ploidy status of the trial individuals. Keeping this in mind triploid and diploid samples were re-evaluated using flow cytometry and microsatellite markers. The individuals that were assessed initially, along with the individuals from the two canning-yield trials, can be seen summarized in Table 3.3.

**Table 3-3** Results from the two methods of verification during the initial verification of triploidy throughout Phase 2.

Group	Age at Sampling	Treatment	Ploidy Verification Results
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			Molecular Markers	Flow Cytometry
A	52 months	Triploid	28 out of 38	No Result
		Diploid	6 out of 8	No Result
B	49 months	Triploid	Not Sampled	No Result
		Diploid	Not Sampled	No Result
C	46 months	Triploid	Not Sampled	No Result
		Diploid	Not Sampled	No Result
Canning 1	56 to 62 months	Triploid	17 out of 18	No Result
Canning 2	60 to 66 months	Triploid	18 out of 18	No Result

The initial results of molecular microsatellite markers showed that not all individuals marked as triploid were indeed aneuploid. Unfortunately, no result could be obtained from flow cytometry to confirm this result. Flow cytometry has been applied successfully in many species to verify or identify triploidy, including *Haliotis midae*. In the two studies preceding this one, both De Beer (2004) and Schoonbee (2008) used flow cytometry to verify triploidy. In this study, however no credible result could be obtained using this method of validation.

Previously flow cytometry was successfully applied using gametes (De Beer, 2004), larvae (De Beer, 2004, Schoonbee, 2008) and juvenile abalone (Schoonbee, 2008). Non-destructive sampling was desired and the large, differentiated epipodial sample resulted in too much background noise during analysis. All efforts to clean and refine these results were unsuccessful and flow cytometry could therefore not be used as a verification tool in this study. This made it impossible to compare flow cytometry to molecular markers as a biotechnological tool for ploidy validation in triploid genotypes from the species *H. midae*. Consequently, flow cytometry will not be included in any of the analyses below.

Twenty-eight of 38 samples (73.68%) marked as triploid were identified and confirmed as aneuploid using microsatellite markers. For the diploid confirmation only 6 out of 8

(75%) individuals marked as diploids could be confirmed using the molecular markers. Further analysis during the two canning trials yielded 17 out of 18 (94.44%) aneuploidy in canning trial one and an 18 out of 18 (100%) aneuploidy in canning trial two.

From these results, it is concluded that not all individuals in the experiment have genotypes that corresponds to the specified experimental treatment, either triploid or diploid. This result was unexpected, considering that diploid individuals would not be expected to revert to an aneuploid state. Two possible explanations for this unexpected result can be considered. The first is that during the original marking process, a triploid individual was incorrectly marked as diploid and entered the trial as a diploid. The second has to do with the refinement of the molecular markers used. During the identification of possible alleles that could be used, some individuals may have more than two copies of a specific allele. When that specific allele is used as a molecular marker, the individual may show more than two alleles, and appear to be triploid. This is the more likely scenario and this problem can only be eliminated with further development and refinement of the molecular marker system.

The percentage of aneuploidy or diploidy obtained in this study was still considered high enough to draw reliable conclusions from the trial animals based on ploidy. Individuals marked as triploid or diploid can therefore be considered accurate based on their designated physical tags. In similar studies regarding triploid Halibutids not all individuals who had undergone triploid induction was verified as aneuploid later in the individual experiments (Stephens and Cook, 1998; Schoonbee, 2008). The possibility of reversion to the diploid state remains innate in aneuploid individuals, but at worst 73.68% of individuals marked as triploid showed aneuploidy. Unfortunately, diploid individuals did not show 100% euploidy either, resulting in the conclusion that the method needs to be refined further. Any changes in the accuracy of the verification method will most likely result in an increased aneuploidy measured in these experimental individuals.

#### **b. Determination of ploidy state of unidentified individuals**

Schoonbee (2008) marked the experimental animals with plastic tags. Due to the environment and abrasive nature of the test subjects, many of these tags were lost. This

results in an unknown individual (ploidy state unknown) and the individual becomes a non-participant in the experiment.

During the two phases of this experiment, new tagging methods were investigated and a more effective tagging method has been developed. This method utilizes plastic bee tags stuck onto the shell with glue. A tagged or marked individual can now be successfully marked for a longer period of time (Brink, *et al.*, 2009).

At the time of verification of ploidy status (46 months onward), many individuals had lost tags and became non-participants which resulted in the experimental group decreasing in numbers. It was then decided that using flow cytometry or molecular markers to identify newly marked individuals would increase the size of the experimental population. Coloured bee tags with a number (one to 99) allowed individuals to be marked and then sampled and the ploidy status of these individuals to be established. Once the individual's ploidy status was determined, it could again be used in the trial.

Ninety-nine individuals were randomly selected and sampled during three different age periods (46, 49 and 52 months of age). These samples were then prepared for analysis using either flow cytometry or molecular markers according to each specified protocol. The results of the ploidy determination can be seen in Table 3.4. The outstanding numbers in each repeat are the individuals that were never identified because no result could be obtained using flow cytometry.

If the assumption is made that tag loss and mortalities are random, and equal numbers of triploid and diploid individuals were entered into the trial, a random sample will yield a 50% triploid to diploid ratio.

**Table 3-4** Triploid percentage in a randomly selected group of ploidy unknown individuals using molecular markers.

Group	Age at Sampling	n	Ploidy		Percentage Triploidy		Chi-Square
					Result	Expected	
A	52 months	88	Triploid	42	44.4%	50.0%	0.63 p < 0.001
			Diploid	45	55.6%	50.0%	

B	49 months	85	Triploid	40	47.1%	50.0%	0.17 p < 0.001
			Diploid	45	52.9%	50.0%	
C	46 months	63	Triploid	28	47.7%	50.0%	0.11 p < 0.001
			Diploid	35	52.3%	50.0%	

From the results above it becomes apparent that this was indeed true for this trial and that the experimental population had a 50% triploid to diploid ratio (Chi-square values all suggest that the observed result is the same as the expected result). The numbers on the uniquely coloured tags also allowed many individuals to be re-entered into the trial.

Molecular marker analysis can therefore be considered a reliable method to identify the ploidy status of unknown (lost tag) individuals within the trial.

### **c. Comparison of flow cytometry to molecular markers for suitability of aneuploidy verification in *Haliotis midae***

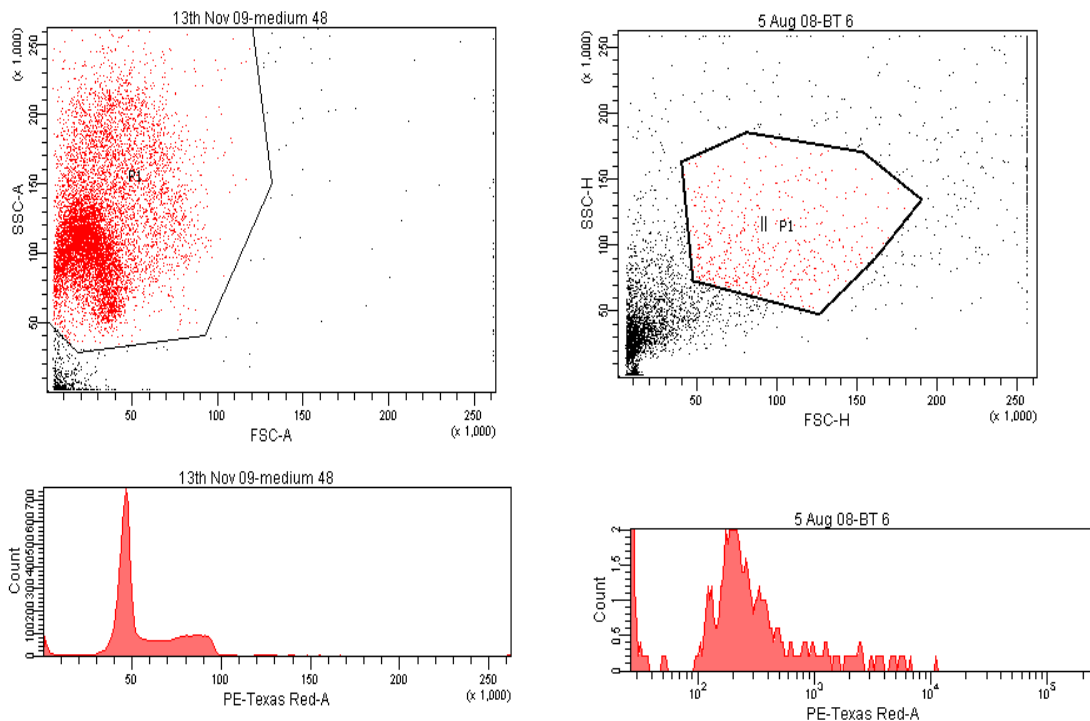
Flow cytometry was originally used since it allows the quick validation of many samples. It was also investigated since it is a cost effective method to confirm triploidy. For this trial, however non-destructive sampling was required. Flow cytometry has previously been used to confirm triploidy in experiments including the Blacklip abalone, *Haliotis rubra* (Liu, *et al.*, 2004; 2009), the tropical abalone, *H. asinina* (Norris and Preston, 2003), Pacific abalone, *H. discus hannai* (Zhang, *et al.*, 1998) and Perlemoen, *H. midae*, (Stepito, 1997; De Beer, 2004; Schoonbee, 2008).

In the above trials the sampling was destructive with gills, blood and gametes all providing useful tissues for analysis using flow cytometry. In the current study non-destructive sampling was however required. Epipodia can be cut off live specimens and used for analysis without having to kill the individual.

In the experiment preceding this one by Schoonbee (2008), both two-day-old larvae and epipodia were used in conjunction with flow cytometry to confirm triploidy. During this current phase of the trial, there were various difficulties in obtaining useable results with flow cytometric analysis.

Originally, Schoonbee (2008) used propidium iodide to lyse and stain the cells, and positive results were achieved. In this part of the trial however, no results could be gathered using the preceding method. Hence, it was finally decided that propidium iodide was not a strong enough enzyme to isolate nuclear cells from the tough epipodial samples.

This led to the use of a DNA kit specifically designed to isolate and stain nuclei, but again unsatisfactory results were gathered. After many trial and error adjustments, it was decided to abandon flow cytometry and utilize molecular markers. After deliberation with the flow cytometry technician, it was concluded that the numerous cell types in a single mature epipodium sample caused the inability to isolate a cell population for analysis. In Figure 3.2 below it can be seen what a sample of isolated cell nuclei look like when displayed as a histogram during flow cytometry analysis. Also, note the unusable result with cell debris and other nuclei that cause the so-called background noise.



(a.)

(b.)

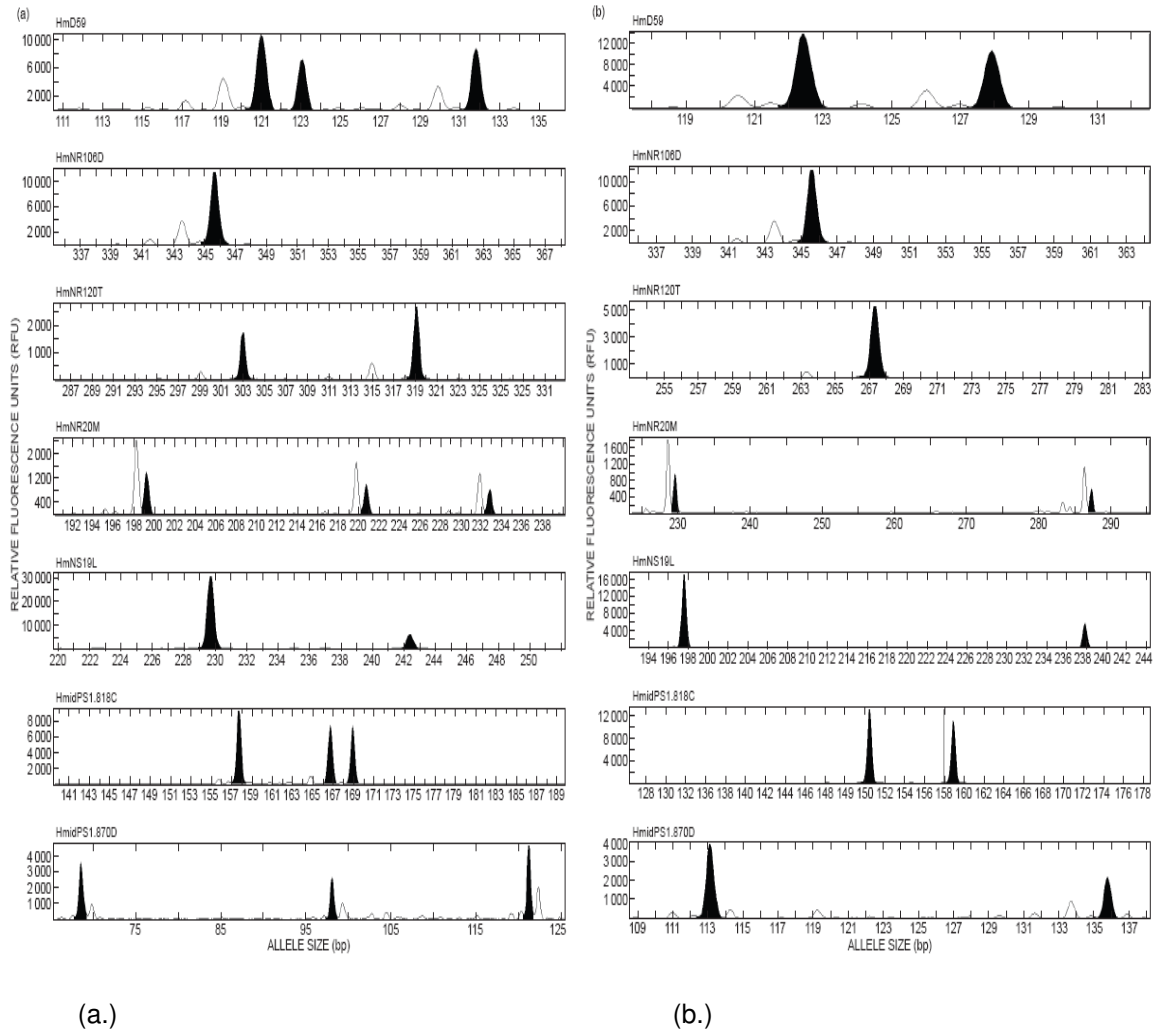
**Figure 3-2** Flow cytometry results for *Haliotis midae* with (a) showing a successful result and (b) showing an example of the unsuccessful results obtained during this study.

d a  
and

noise is cells or debris that is either larger or smaller than the cell nuclei in the desired sample, and can include proteins, cell materials and other nuclei.

Molecular marker analysis is a new method, and markers need to be specifically designed for a specific species. Through the Innovation Fund, the Molecular Aquatic Research Group of the Genetics Department of Stellenbosch University developed and used 30 microsatellite loci to genotype the 16 control samples. Fluorescent analysis was performed with a 3730xl DNA Analyzer (Applied Biosystems) and genotyping with GeneMapper version 4 (Applied Biosystems) as described in Slabbert (2010b). The markers chosen for the triploidy verification multiplex were reviewed and seven unique markers were selected for the final verification multiplex. Slabbert (2010b) used eight known triploid and eight known diploid samples to develop the verification multiplex through the protocol described.

Figure 3.3 shows an example of the electropherograms showing the alleles of each of the seven loci used for triploid verification for (a) a triploid and (b) a diploid individual. It has now become possible to cost effectively identify a ploidy unknown individual as either triploid or diploid. There are still some problems related to this method: It is possible for some individuals to have more than two copies of a specific allele and if used as a molecular marker the individual may appear to be triploid. Another problem related to the use of molecular markers is that although it has become much more accessible and less expensive to use, it remains more costly than flow cytometry.



**Figure 3-3** Electropherograms showing the alleles of each of the seven loci used for triploid verification in (a) a triploid and (b) a diploid individual (Slabbert, *et al.*, 2010b).

In Figure 3.3 it can be seen that the triploid individuals showed three alleles in some of the loci with the diploid individuals never showing more than two alleles in any loci. This enables the verification of triploidy in *H. midae*.

### 3.3 Summary

The main objective of this chapter was to confirm triploidy in trial animals and that an individual marked and assessed as triploid was indeed aneuploid. It was deemed important since the possibility of reversion remains inborn in biological systems and the

long production period for *Haliotis midae* increases the chance that this occurs. Molecular marker analysis showed that if there was reversion it remained relatively low and an aneuploid individual can be considered to remain in this state for the duration of the production period.

A second aim achieved in this chapter was the 50% distribution of triploidy in the unmarked individuals. This has a two-fold advantage; the ploidy state does not negatively affect the survival of *Haliotis midae* and if an individual has lost its tag and essentially becomes unknown, it can be assessed using molecular markers and its identity recovered.

Unfortunately, flow cytometry could not be successfully applied during Phase 2 and only molecular markers gave desirable results.

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## **4. A comparative analysis of the growth rate of triploid and diploid genotypes of abalone, *Haliotis midae*, over a period from 30 to 60 months of age.**

### **4.1 Background**

The abalone *Haliotis midae* is the most important aquaculture species in South Africa, valued at R268 million in 2008. South Africa has also developed into the largest producer of farmed abalone outside of Asia (Troell, *et al.*, 2006; FAO, 2010) and is recognized for the development of abalone culture technology (Sales and Britz, 2001), including biotechnology.

Ploidy manipulation has been applied in various commercial aquaculture species, including shellfish, to improve growth rates. The improved growth displayed by triploid genotypes of various commercial aquaculture species arises mainly from the induction of sterility (Liu, *et al.*, 2009). Beaumont and Fairbrother (1991) summarized the benefits associated with triploidy and related sterility in terms of:

1. Energy used for gametogenesis is diverted to somatic growth allowing the triploids to display faster growth.
2. The prevention of disadvantages associated with the development of mature gonads and uncontrolled spawning during transport to sales.
3. Reduction of the risks of farm stock to natural biosecurity.

Stepito (1997) induced triploidy in abalone (*H. midae*) through Cytochalasin B treatment and subsequently reported improved growth, though not statistically significant, compared to diploid controls up to an age of 550 days (18 months). Zhang *et al.* (1998) reported that triploid genotypes of the Pacific abalone (*H. discus hannai*) displayed similar to slightly superior growth rates when compared to diploid controls, though not significant in most cases or very small. Zhang *et al.* (1998) also reported that higher levels of heterozygosity were observed in triploids and this, along with the possible selective effects of the harsh chemical treatment (6-DAMP) eliminating weaker individuals, could explain the superior performance of the triploids in that trial.

The advantages of triploidy are however not confined to growth, and the benefits associated with sterility, referred to by Beaumont and Fairbrother (1991), should be considered when evaluating triploidy. This is evident from studies by Liu *et al.* (2004) and Liu *et al.* (2009) on *H. rubra* that reported abnormal and retarded gonadal development of the triploid individuals in a 30 month experimental period (7 to 37 months of age). Liu *et al.* (2009) also reported that the diploid genotypes had already spawned whilst none of the triploid genotypes did during the spawning season at an age of 34 to 37 months.

Techniques for the induction of triploidy in *H. midae* through the use of hydrostatic pressure was developed (De Beer, 2004) and applied to assess the effect of triploidy on the growth performance and reproductive status. Schoonbee (2008) compared the growth rate of diploid and triploid genotypes of *H. midae* over the period of eight to 24 months of age and reported no significant difference in growth rate based on shell length and body weight. Schoonbee (2008) recommended that the comparative growth analysis be continued beyond the age of onset of sexual development and maturity, at which stage the growth advantages associated with sterility may come into effect.

This chapter reports on the continued assessment of comparative growth rate of diploid and triploid genotypes as described by Schoonbee (2008) over the period of 30 to 60 months of age.

## **4.2 Materials and Methods**

### **4.2.1 Material**

This study was conducted at the Danger Point Irvin and Johnson (I&J) Abalone Culture Division, near Gansbaai in the Western Cape. In 2006, three distinct progeny groups were produced at quarterly intervals in accordance with a procedure described by Schoonbee (2008). For each of the three spawning events, 15 females and 10 males were randomly selected from the brood stock at the I&J Gansbaai abalone hatchery. The males and females were induced to mass spawn using standard hatchery procedures as described by Schoonbee (2008).

The eggs were collected by siphoning them from the bottom and 200 mL concentrated eggs, filtered through a 400  $\mu\text{m}$  sieve, immersed in 17°C seawater was fertilized with 25 mL of sperm using standard fertilization procedures.

Three to four fertilizations and subsequent triploid inductions had to be performed during each spawning event due to the 150 mL limited capacity of the pressure induction equipment.

After fertilization, the zygotes were collected and placed into the pressure vessel. The vessel consists of a stainless steel cylinder with a volume of 150 mL and a headpiece, that seals with a rubber o-ring, with a pressure gauge and valve. The vessel fits into a steel frame so that the cylinder and headpiece can be clamped between the top of the frame and a hydraulic jack at the bottom to apply the required pressure.

Pressure was applied to the zygotes according to the recommendations by De Beer (2004). A sample was taken 48 hours after fertilization to verify the ploidy status of both treated and untreated larvae using a flow cytometry method described by De Beer (2004). De Beer subsequently reported high levels (near 100%) of triploidy of the treated groups of larvae. The ploidy status of both diploid and triploid treatment groups were again verified at 26 months of age, with the triploid group displaying near to 93% levels of aneuploidy (expected to be functionally sterile) (Schoonbee, 2008).

The treatment groups were then reared in a manner as described by Schoonbee (2008) and tagged at seven months of age, with the smallest individuals at 5 mm in length. They were then entered into a standardized growth trial described by Schoonbee (2008) with measurements for shell length and body weight recorded at three-month intervals up to the age of 24 months.

#### **4.2.2 Experimental design**

A Random Block Design was used as an experimental design. The three spawnings conducted at quarterly intervals was used to establish three Blocks (A, B and C). Each Block contained a diploid and triploid Treatment group. Animals from each Treatment group were randomly divided into three Repeats (Table 4.1), with 350 individuals per

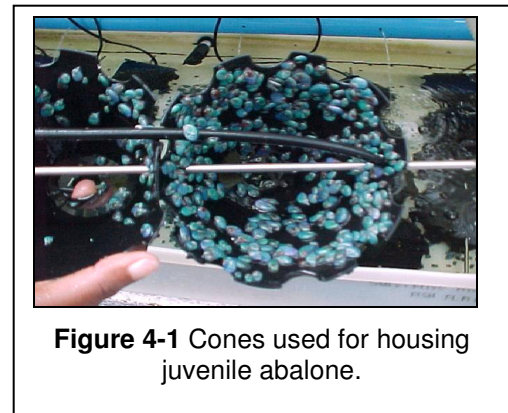
Repeat, as described by Schoonbee (2008). Each individual was marked using a specific colour coded tag to distinguish between Treatments (triploid or diploid) as well as between Repeats.

**Table 4-1** The Random Block experimental design as used for the comparison of growth rates of diploid and triploid abalone, *Haliotis midae* (n = number of animals per treatment at start of trial).

Groups (A, B and C)	Repeat 1	Repeat 2	Repeat 3
Treatment 1: Diploid	n = 350	n = 350	n = 350
Treatment 2: Triploid	n = 350	n = 350	n = 350

Abalone are on-grown in land-based tanks in most of the abalone farms in South Africa. The tanks are usually rectangular with typical dimensions of five meters (L) x two meters (W) x one meter (H). They contain baskets, which in turn are made up of a number of plates, to which the abalone attach themselves. Tanks are aerated to replenish the dissolved oxygen in the water of the tanks, to remove carbon dioxide from the water and to increase water quality homogeneity through mixing (personal communication, Gert le Roux, 2009).

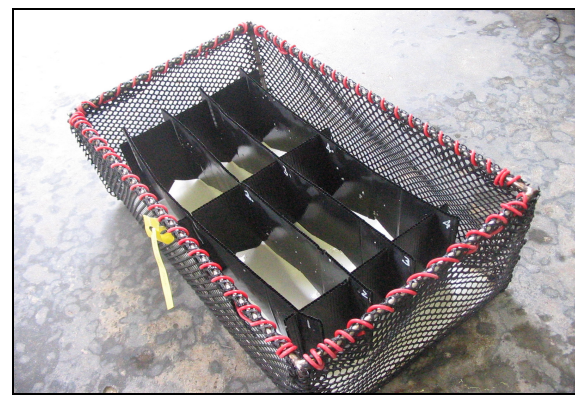
Two types of housing systems were used during the 62-month trial period (Phase 1 and 2). A tray system was used for the housing of the animals up to nine months of age, including the first two measurements at eight and nine months, respectively. Each tray contained six specific habitats, in the shape of cones, under which the abalone could find shelter (Figure 4.1).



**Figure 4-1** Cones used for housing juvenile abalone.



The animals were then transferred to a basket holding system, seen in Figure 4.2, at age nine months to reduce stocking densities and competition amongst animals and to improve the ease of handling, maintenance and feeding. The animals were maintained in such baskets for the remainder of the experimental period. Each basket contained a corrugated sheet grid that provided four



**Figure 4-2** Basket housing system used for commercial abalone production.

panels as substrates as explained by Schoonbee (2008). Schoonbee (2008) concluded measurements up to the age of 24 months and reported on the comparative growth performances of the treatment groups during the first growth phase.

The experiment then continued in the current study for a second growth phase with measurements of shell length and body weight recorded at three monthly intervals from the age of 29 to 62 months.

The animals were fed *ad libitum* throughout the second growth phase with a combination (30: 70 ratio) of Abfeed (supplied by Marifeed Pty Ltd) and kelp (*Eclonia maxima*) according to standard farm practices.

The tanks containing the baskets were routinely cleaned to prevent the accumulation of benthic or sessile organisms. The stocking density was maintained at commercial levels (6-8 kg of abalone per basket) by randomly splitting the baskets at required intervals of approximately every six months. Equal numbers of triploid and diploid individuals were maintained at each split.

### 4.2.3 Feeding and growth

In abalone 63% of the energy gained from food is excreted as faeces, 32% is used for respiration and 5% for growth and reproduction (Barkai and Griffiths, 1988). Most of the energy allocated to growth and reproduction in juveniles is used for somatic growth. As

soon as maturity is reached, energy becomes less available for somatic growth since it is allocated to reproduction.

Growth in juvenile abalone is mainly dependent on the type and availability of food and is affected by competition, management and water quality (Day and Fleming, 1992). Abalone are erratic feeders and are sensitive to environmental and physiological stimuli. These stimuli result in fluctuating feed intake and growth rates due to energy spent on stress responses (Huchette, *et al.*, 2003). An important objective of abalone farming is to maintain a constant, good growth rate by ensuring optimal environmental and husbandry conditions to sustain proper feeding (Flemming, 1995; Schoonbee, 2008).

Another factor to consider in abalone growth is competition that arises directly from stocking density and subsequent availability of feed. The availability of feed depends on the amount of feed provided per abalone in a housing unit as well as the ability of each abalone to reach the feed. During a comparative growth trial, it is therefore important that factors such as stocking densities, feeding regimes, water and air supply and the handling of the animals are kept constant for all the Treatments and Groups (Schoonbee, 2008). These considerations were applied to this specific experiment in the current study.

#### **4.2.4 Ploidy determination**

It was considered important to verify the ploidy status of the treatment groups at various stages throughout the trial period (Schoonbee, 2008). The initial success of triploid induction was verified using two-day-old larvae. The ploidy status of treatment groups was reassessed at 26 months of age and again at 46, 50 and 53 months of age during this trial as described in Chapter 3. The procedures used for verification of ploidy status of the two day old larvae and reassessment after the first growth phase at 26 month is described by Schoonbee (2008).

A final verification was also conducted after the second growth phase at 66 months of age. For this analysis, as well as for verification at 46, 50 and 53 months of age epipodia tissue samples were taken and preserved for flow cytometry in Eppendorf

tubes in an mPBS and DMSO solution, frozen at -20°C and in 99% (v/v) ethanol for molecular marker analysis. A detailed discussion of the verification process is presented in Chapter 3. Once the triploid or diploid individual loses its tag it becomes an unknown individual and cannot be included in the trial. The diploid individuals were used as controls during the verification process. Apart from an approximate 30 tagged triploids and diploids for reassessment, an additional 100 individuals displaying tag loss were randomly selected from each Repeat for inclusion in ploidy assessment. They were retagged using a plastic bee tag with a corresponding number in order to trace back their respective ploidy status after verification.

#### **4.2.5 The shell**

The condition of the shell of abalone is a direct indicator of the general health and well-being and individuals with damaged shells show severe and often permanent retardation in growth. Since energy is spent in trying to correct the damage, less energy is spent on growth. The following aspects relating to the condition of shells of animals should be considered for inclusion into growth trials:

1. Animals with damaged shells should not be included into growth trials.
2. Animals that show large differential rates of damage / growth should not be compared to each other, either within replicates or between batches (Schoonbee, 2008).

#### **4.2.6 Tagging**

Tagging of abalone, juveniles in particular, poses a challenge in that the tagging method should ensure a durable, long lasting (one to two years) tag and does not impose stress or hindrances on the animals. Most common methods are based on attaching a tag to the shell with an adhesive or by attaching the tag to a shell pore. Studies have shown that attaching the tag to the shell pore causes stress and interferes with growth patterns (Newman, 1968; McShane, *et al.*, 1988). A soft silicone tag inserted into the first

respiratory pore proved unreliable due to high levels of tag loss in a short period (Vorster, 2003; Schoonbee, 2008).

The tagging method used in this experiment was similar to the one used by the South African Innovation Fund Abalone Project. Super Glue™ is used to attach a small plastic numeric colour coded tag (bee tag) in-between the shell ridges (personal communication, Arnold Vlok, 2007; Schoonbee, 2008). This method displayed good adherence and although limited tag loss still occurred, the tags remained on the animals for long enough to allow retagging at a later stage. As the animals increase in size over time, tag loss tends to increase. This is attributed to the more abrasive behaviour of the larger animals under increased densities and higher levels of competition for food, resulting in increased stacking of the abalone on top of each other (Huchette, *et al.*, 2003). Such behaviour tends to increase the occurrence of tag loss.

All animals from each treatment group were tagged at the onset of each growth phase, whilst retagging was also conducted during the growth phase to maintain integrity of the group identifications. The tagging method used is described by Brink, *et al.* (2009). The animals were anaesthetized during measurements and spread out on a sponge with their shells facing upward. Excess water was removed from their shells using a paper towel and allowed to air dry for a couple of minutes. A small drop of Super Glue™ was placed on the shell underneath the whorl with care taken not to allow glue into the breathing pores. A coloured plastic bee tag was then placed onto the drop of glue and left to dry. All the abalone were then placed back into their baskets.

The method using Super Glue™ and plastic bee tags have a low tag loss percentage but losses do occur. This loss of tags results in the individuals becoming ploidy-unknown and they effectively fall out of the experiment. It is important to exclude any unknown individual from the experiment. Occasionally animals suffering tag loss were retagged, followed by reassessment of the ploidy status and reassignment to the respective treatment group, in order to maintain sufficient numbers of identifiable individuals per Repeat. A detailed discussion on the reassignment of animals to their groups of origin is presented in Chapter 3.

## 4.2.7 Sampling and Measurements

A total of 17 measurements were taken during this 30 month trial period, at the exact ages of 29, 33, 36, 37, 39, 40, 42, 43, 46, 49, 50, 51, 53, 55, 58, 59 and 62 months. Measurements for individual shell length (millimetre) and body weight (gram) were taken on a total of 16 randomly sampled individuals of each treatment group from each Repeat at each interval. The measurements were taken in a non-destructive manner after which animals were placed back in the group of origin.

Measurement of shell length was taken along the longest diagonal with a Vernier Calliper while body weight was measured on a 200 g x 0.01 g calibrated electronic scale.

A standardized random sampling method was used during which all animals from a particular Repeat were anaesthetized and spread out evenly on a sponge that soaked up excess water and slime. A string was then placed over the batch to create a transect (Figure 4.3). Starting from one side of the transect, diploid and triploid individuals that touched the transect line was sampled until the required number



**Figure 4-3** Sampling abalone along a transect.

of 16 individuals per treatment group were obtained. If the transect was completed with animals from a particular treatment group still outstanding, a new transect line would be placed perpendicular to the first one and the procedure repeated until the required number was obtained.

## 4.3 Results and Discussion

The main objective of the study was to assess if there was a differential growth rate between diploid and triploid genotypes of the abalone, *Haliotis midae*, in the growth period of 29 to 62 months, during maturation.

Secondary objectives were to establish the relationships between growth traits over time. Since Schoonbee (2008) showed there was no effect of seasons on the growth rate this was not investigated further in the current study and the Blocks were used as Repeats.

### 4.3.1 Data analysis

Individual body weight (wet weight measured in grams) and shell length (measured in millimetres) was collected at regular three monthly intervals during the 33-month period. This resulted in data for the analysis of growth rate for the age period of 29 to 62 months.

The experiment was a continuation of the work done by Schoonbee (2008) and consequently the experimental layout remained a Random Block Design. The experimental layout consisted of one Block, with each Block containing three Repeats of two Treatments; diploid and triploid. For full detail on the Random Block Design, refer to section 4.2.2. In the study by Schoonbee (2008), the Blocks were related to season and were based on separate spawnings in three consecutive seasons (summer, winter and spring). Since Schoonbee (2008) found no effect of season on growth this was not investigated further in this analysis of growth and each Block became a Repeat.

The data was then analyzed in a progressive manner to:

- a. Assess the effect of ploidy on the average growth performance, ignoring seasons, by means of a covariate analysis.
- b. Compare the growth performance of diploid and triploid groups over Blocks by means of regression analyses for length and weight on age.

- c. Describe the weight-length relationship for triploid and diploid groups during Phase 2 (29 to 62 months) and Phase 1 and 2 combined (8 to 62 months).

#### 4.3.1.1 Assessment of the effect of ploidy on average growth performance

Growth rates between triploid and diploid Haliotids in various studies, *H. midae* (Stepito, 1997), *H. discus hannai* (Zhang, *et al.*, 1998), *H. rubra* (Liu, *et al.*, 2004; 2009) to mention but a few, show varying results in terms of performance. It becomes clear that this growth performance is dependent on the species in question as well as the method for triploid induction.

Based on these discrepancies in growth rates and recommendations by Schoonbee (2008) the growth performance of triploid *H. midae* was compared to diploid counterparts between the age period of 29 and 62 months in an effort to establish and quantify the commercial impact of triploidy in South Africa.

The age-adjusted means were used in a covariate analysis and the length and weight gain over time used to calculate the regression allowing the quantification of the effect of the treatment on the average growth performance in *H. midae*.

#### Assessment of the effect of ploidy on the basis of Adjusted Mean Length

A covariate analysis with age as covariate was performed on the data to compare the mean length over Blocks, ignoring seasons. A difference between these age-adjusted means would indicate an effect of the Treatment (triploidy) on the length gain of abalone. Table 4.2 indicates that there is a statistically significant difference between Treatments based on shell length.

**Table 4-2** Results of the covariate analysis with age as a covariate for length-wise growth differences between triploid and diploid abalone (for p-values > 0.05, the means were considered as similar and for p < 0.05 the means were considered as statistically different from each other).

Source (Model)	df	Mean square	p-value
Age (Regression)	1	292820.87	<0.0001
Ploidy	1	1289.84	0.0015

Error	1291	126.72	
Uncorrected total	1293		

Table 4.3 indicates a difference in the adjusted mean length of abalone. It can therefore be concluded that triploid induction has a statistically significant effect on the consequent growth of abalone in the form of average length. The difference of 1.99 mm over 62 months relate economically to a month of production time. Based on growth in shell length alone it would mean that triploid induction has a significant negative impact on *Haliotis midae* for commercial production.

**Table 4-3** The adjusted mean length of triploid and diploid abalone (*H. midae*) with the results of a pair-wise T test to indicate the similarity of the mean length (mm) (for p-values > 0.05, the means were considered as similar and for p < 0.05 the means were considered as statistically different from each other).

Treatment	Adjusted Mean Length	T test	t-value	p-value
Diploid	76.61 mm	a	3.19	0.0015
Triploid	74.61 mm	b		

### Assessment of the effect of ploidy on the basis of Adjusted Mean Weight

A covariate analysis with age as covariate was performed on the data to compare the mean weight over Blocks, ignoring seasons (Table 4.4). A difference between these age-adjusted means would indicate an effect of the treatment (triploidy) on the weight gain of abalone.

**Table 4-4** Results of the covariance analysis with age as a covariate for weight-gain growth differences between triploid and diploid abalone (for p-values > 0.05, the means were considered as similar and for p < 0.05 the means were considered as statistically different from each other).

Source (Model)	df	Mean square	p
Age (Regression)	1	3238451.41	< 0.0001
Ploidy	1	8500.92	0.0091



Error	1291	1246.67	
Uncorrected total	1293		

Table 4.5 indicates a difference in the adjusted mean weight of abalone. It can therefore be concluded that triploid induction does have a significant effect on the consequent growth of abalone in the form of average weight.

**Table 4-5** The adjusted mean weight of triploid and diploid abalone (*H. midae*) with the results of a pair-wise T test to indicate the similarity of the mean weight (g) (for p-values > 0.05, the means were considered as similar and for p < 0.05 the means were considered as statistically different from each other).

Treatment	Adjusted Mean Weight	T tests	t-value	p-value
Diploid	96.63 g	a	2.61	0.0091
Triploid	91.50 g	b		

The results in Tables 4.4 and 4.5 gave an indication of significant differences between the weights of the diploid and triploid abalone, when assessed over Blocks. The nature of the difference (5.13 g in terms of weight) is both statistically significant and commercially significant. When considering that a typical commercial abalone farm produces 100 tonnes per annum, at an average weight of 150 g per individual, with a loss of 5.13 g per individual, it equates to a loss of 3.42 tonnes per year. With the high farm gate value per kilogram, this weight difference becomes economically significant.

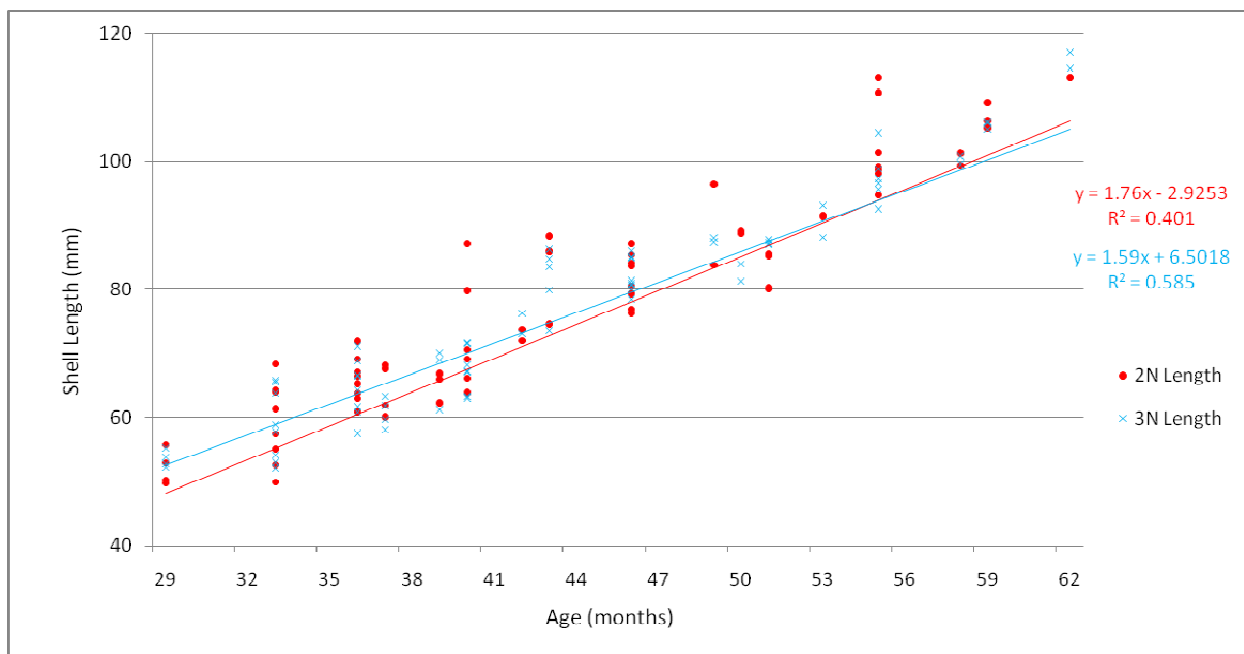
#### **4.3.1.2 Regression analysis of growth performance for diploid and triploid abalone**

In a comparative growth trial, regression is a very useful tool for analysis. It allows the prediction and evaluation of growth between treatments for different age periods, even the assessment of future growth values.

### Regression of length on age, for diploids and triploids, over Blocks

The shell length growth of the triploids and diploids over Blocks was assessed by means of a length on age regression analysis. A linear equation was developed to describe the respective relationship between the length of triploids and diploids and age. The equation was then used in further comparisons between the growth of triploids and diploids.

Figure 4.4 displays the linear regression for triploid and diploid abalone length gain over time.



**Figure 4-4** The linear regression of shell length on age of triploid and diploid abalone, *H. midae* (solid red dots = diploid, blue crosses = triploid). The linear equations as well as the individual R-squared values are displayed on the right.

Quadratic regression resulted in very low R-squared values and was not considered during the regression analysis of length on age.

The respective linear regression equations between length (Y) and age (X) were calculated as:

- Diploid:  $y = 1.76x - 2.9253$

- Triploid:  $y = 1.59x + 6.5018$

with R-squared values of 0.401 and 0.585 respectively as indication of average fits to the data.

Application of these equations to predict the respective length of abalone at 12 months and 56 months provided the following results, which showed no economically significant differences between triploid and diploid variants, based on the regression of shell length on age.

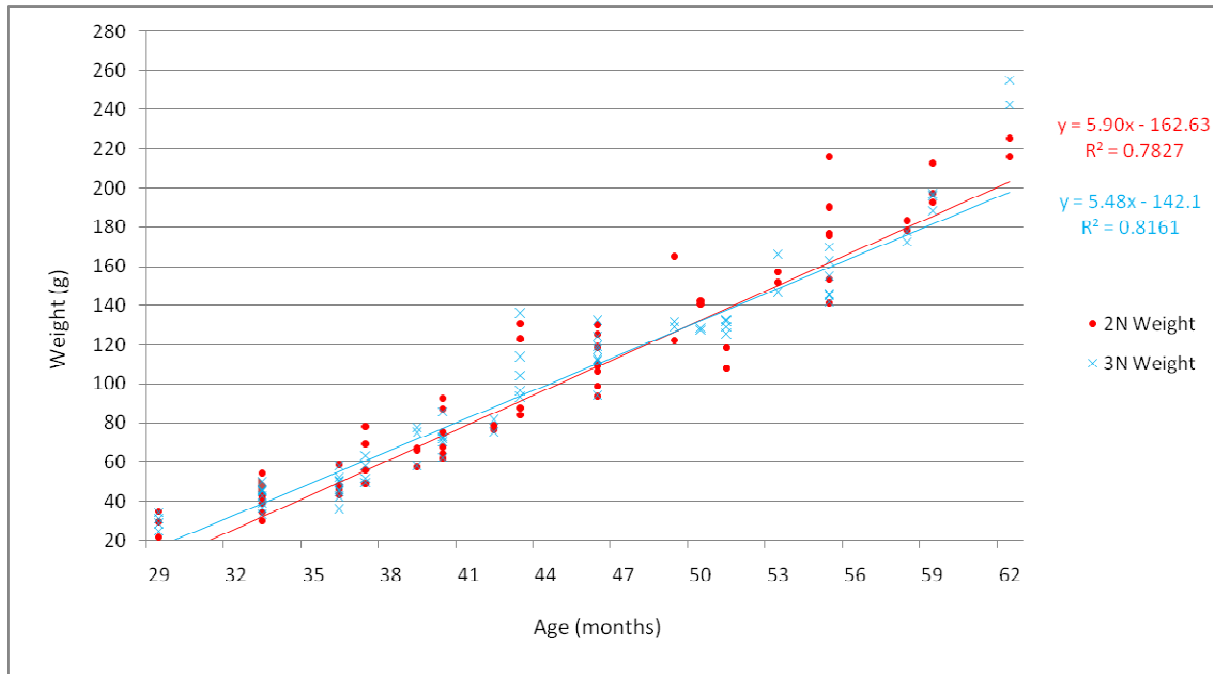
Average length at age 12 months:	Diploid	= 18.19 mm
	Triploid	= 25.58 mm
Average length at age 56 months:	Diploid	= 95.63 mm
	Triploid	= 95.54 mm

The nature of the difference between triploid and diploid abalone for the 12-month prediction result from the large discrepancies between the y-axis intercepts. The diploid abalone showed a faster growth rate compared to the triploid abalone based on the data and linear regression.

### **Regression of weight on age, for diploids and triploids, over Blocks**

The wet weight growth of the triploids and diploids over Blocks was assessed by means of a weight on age regression analysis. Both linear and quadratic equations were developed to describe the respective relationships between the length of triploids and diploids and age. The best fitting equation was then used in further comparisons between the growth of triploids and diploids.

Figure 4.5 displays the linear regression for triploid and diploid abalone weight gain over time.



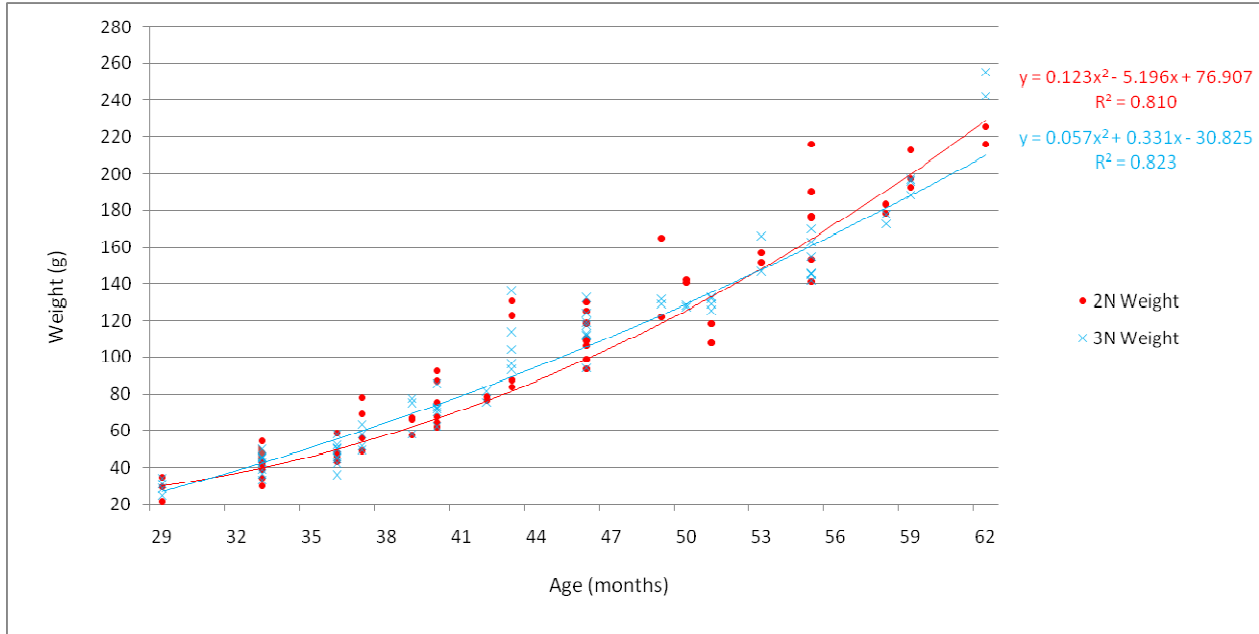
**Figure 4-5** The linear regression of wet weight on age of triploid and diploid abalone, *H. midae* (solid red dots = diploid, blue crosses = triploid). The linear equations as well as the individual R-squared values are displayed on the right.

The respective linear regression equations between weight (Y) and age (X) were calculated as:

- Diploid:  $y = 5.90x - 162.63$
- Triploid:  $y = 5.48x - 142.10$

with R-squared-values of 0.783 and 0.816 respectively as indication of good fits to the data.

After the linear regression, a quadratic regression was used to analyse the data. Growth increase in abalone based on weight is expected to be quadratic and the reason for the second regression analysis (Mgaya and Mercer, 1995). Figure 4.6 displays the quadratic regression of weight on age for triploid and diploid abalone.



**Figure 4-6** The quadratic regression of wet weight on age of triploid and diploid abalone, *H. midae* (solid red dots = diploid, blue crosses = triploid). The quadratic equations as well as the individual R-squared values are displayed on the right.

The respective quadratic regression equations between weight (Y) and age (X) were calculated as:

- Diploid:  $y = 0.123x^2 + 5.196x + 76.907$
- Triploid:  $y = 0.057x^2 + 0.331x - 30.825$

with R-squared values of 0.810 and 0.823 respectively as indication of good fits to the data.

The quadratic regression gave higher R-squared values, which suggest a better fit to the data. This is in line with what was expected for weight increase. All further analysis were therefore based on the quadratic regression of wet weight on age for triploid and diploid abalone.

Application of these equations to predict the respective weight of abalone at 29 months, the beginning of Phase 2, and 56 months provided the following results, which showed significant differences between triploid and diploid variants based on the regression of wet weight on age triploid induction.

Average weight at age 29 months: Diploid = 29.67 g

Triplod = 26.71 g

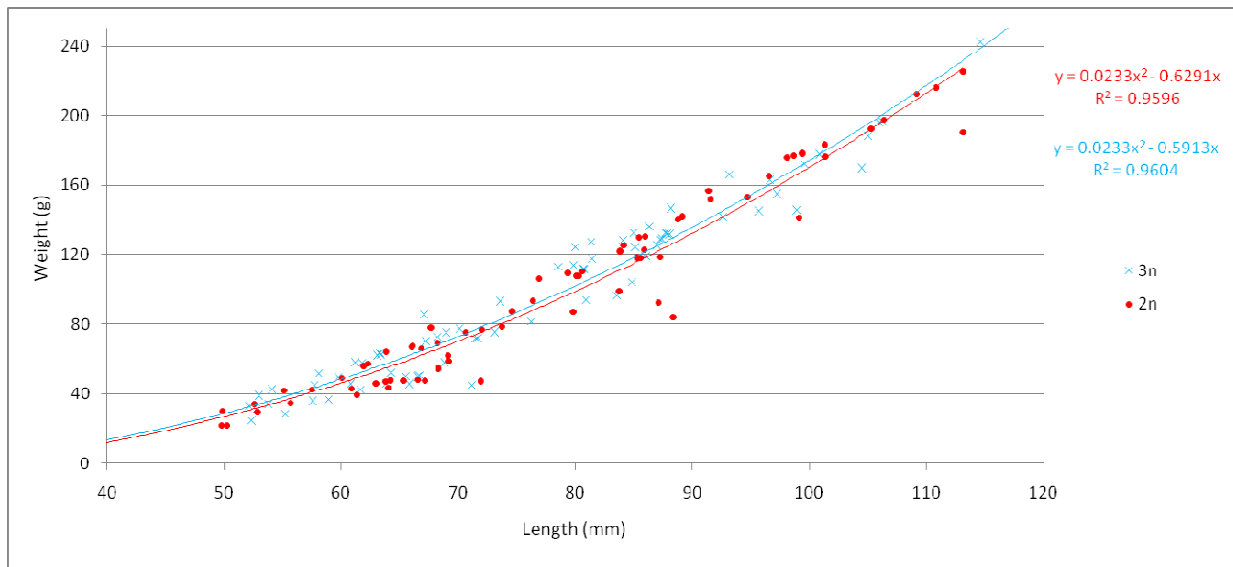
Average weight at age 56 months: Diploid = 171.66 g

Triplod = 166.46 g

It can thus be concluded that, over blocks, significant differences were detected between diploid and triploid weight gain.

#### 4.3.1.3 The weight to length relationship of triploid and diploid variants of *Haliotis midae*

The regression of length on weight gives an indication of the condition index of abalone. This is a useful management tool since it allows for the prediction of one parameter such as weight to be calculated using the other, in this case length. It is easier during commercial production of abalone to measure the length of the abalone, and the weight can then be predicted using the relationship of weight and length. This relationship for Phase 2 is presented in Figure 4.7 below.



**Figure 4-7** The relationship between the mean length and weight of *Haliotis midae* over Treatments (diploid and triploid) for the age period of 29 to 62 months. Solid red dots = diploid, blue crosses = triploid. The quadratic equations as well as the individual R-squared values are displayed on the right.

Separate quadratic regressions were calculated for diploid and triploid Groups and the relationship between length and weight over time is described by the following equations:

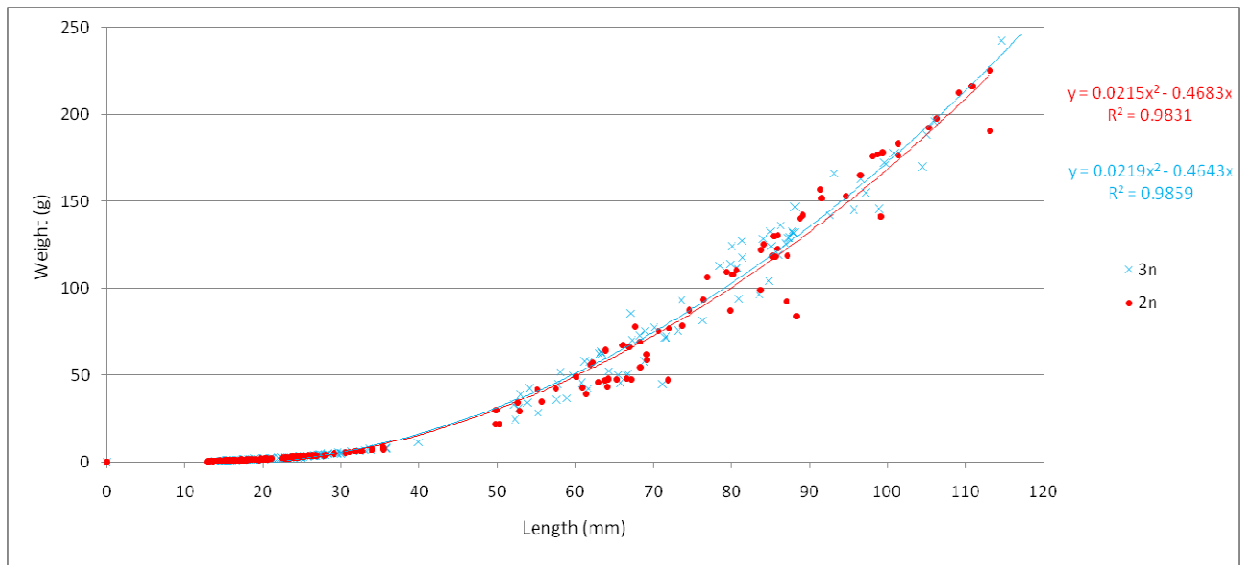
Diploid:  $Y = 0.0233x^2 - 0.6291x$

Triploid:  $Y = 0.233x^2 + 0.5913x$

where Y = weight in grams, X = length in mm and N = 150.

The R-squared values of 0.960 for both regressions indicated a very good fit to the data. The Y-intercepts for both exponential equations were set to zero. This was done since growth does not start at a negative and it did not have any influence on the R-squared values.

Figure 4.8 shows the regression of weight on length for the period of 8 to 62 months. This gives a condition index for both Phase 1 and 2 and gives a clear illustration of the exponential nature of growth in *Haliotis midae*.



**Figure 4-8** The relationship between the mean length and weight of *Haliotis midae* over Treatments (diploid and triploid) for the age period of 8 to 62 months. Solid red dots = diploid, blue crosses = triploid. The quadratic equations as well as the individual R-squared values are displayed on the right.

Again, separate quadratic regressions were calculated for diploid and triploid Groups and the relationship between length and weight over time for Phase 1 and 2 is described by the following equations:

$$\text{Diploid: } Y = 0.0215x^2 - 0.4683x$$

$$\text{Triploid: } Y = 0.0219x^2 - 0.4643x$$

where Y = weight in grams, X = length in mm and N = 397.

The R-squared values of 0.983 and 0.986 for the diploid and triploid regressions respectively indicated a very good fit to the data. The Y-intercepts for both quadratic equations were again set to zero.

The weight over length regression for both phases indicates that there was not a large difference in growth between triploid and diploid variants during this trial.

## 4.4 Summary

This chapter aimed at assessing the effect of triploidy on the growth performance of triploid and diploid genotypes of the species, *Haliotis midae*, based on shell length and wet weight. A covariate analysis was calculated on the average growth performance as well as a regression analysis of growth (length and weight) on age. Finally, the weight to length relationship was described for Phase 2 as well as both Phases 1 and 2 (period of eight to 62 months).

This aim was successfully achieved and the data analysis above indicates the effect of ploidy on the growth performance of triploid abalone (*H. midae*) compared to their diploid counterparts. The results above indicate that based on covariate and regression analysis the triploid treatment reduces the growth performance of *H. midae*.

The weight-length relationship during the two growth phases confirmed that triploid and diploid variants of the trial showed similar and expected growth and that a normal growth curve was achieved. This makes it possible to conclude that growth in triploid *H. midae* was normal but at a reduced rate when compared to diploid abalone of the same species. Whether this reduced growth rate is economically acceptable based on the



other advantages associated with sterility, or more likely, reduced gonadosomatic performance, remains to be seen and is evaluated in the next chapter.

## 4.5 References

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## **5. Assessment of production parameters in triploid and diploid variants of the South African abalone, *Haliotis midae*.**

### **5.1 Introduction**

Abalone are exclusively commercially harvested for their large muscular foot that makes up almost a third of the animal's total wet weight. The demand for this foot muscle in the Far East has led to the increased commercial and recreational exploitation of the species (Tarr, 1993; Godfrey, 2003).

In commercial culture operations the abalone are fed an artificial diet. Because of the expensive nature of the feed and the high production cost for the rearing of abalone on land, it becomes critical to produce animals with high yield percentages and that attain low feed conversion ratios (FCR). FCR is calculated as the mass of food eaten divided by the body mass gain over a specified period of time (personal communication: Mr. Izak Otto, February 2007). By improving these parameters, a commercial operation will improve profitability.

In this study triploidy was investigated as a method to improve growth in triploid genotypes of the South African abalone, *Haliotis midae*. The "triploid advantage" theory suggests that the individuals are sterile and energy used for gametogenesis in diploid genotypes is available to support somatic growth in triploid genotypes (Tabarini, 1984; Allen, *et al.*, 1986; Barber, *et al.*, 1991; Ruiz-Verdugo, *et al.*, 2000).

Triploidy does not necessarily produce complete sterility in shellfish. It more likely produces a decreased or retarded gonadal development. Functional gametes and even spawnings have been recorded in triploid Pacific abalone, *H. discus hannai* (Li, *et al.*, 2004). More recently Liu *et al.* (2009) indicated that triploid variants of the Blacklip abalone (*H. rubra*) had retarded gonadal development and in contrast to their diploid siblings did not spawn during the 30 month experimental period. In the work done by Schoonbee (2008) in the experiment preceding this one, there were no differential growth rates between triploid and diploid siblings and if complete sterility cannot be achieved in triploid *H. midae*, perhaps retarded gonadal development will show enough

of an advantage to justify the induction. Triploid abalone may develop functional gametes, but at a much lower rate thereby limiting their spawning capacity.

Variation in all of the results from the above studies may however simply be explained as a result of differences in food availability (Racotta, *et al.*, 2008), and experimental design therefore becomes important to minimize population variation.

### **5.1.1 Effect of triploidy on processing yield**

Sexual maturation affects flesh quality in molluscs by diverting energy in the form of glycogen to reproduction. Sterility by triploid induction affects body morphology, processing yields and flesh quality (Piferrer, *et al.*, 2009). Triploids may however have similar yields to diploids depending on the species, size and age at maturity (Sheehan *et al.*, 1999).

Gametogenesis in molluscs usually involves the mobilization of reserves from the foot muscle to the gonad. However, in high-food environments such as in commercial culture, such energy transfer may not be required. If this is the case, the foot muscle weight and biochemical composition in triploids may be the same as in diploids and no triploid advantage will exist (Piferrer, *et al.*, 2009). This was observed in the lion-paw scallop, *Nodipecten subnodosus*, by Racotta *et al.* (2008). They also identified the possible transfer of highly unsaturated fatty acids from the foot muscle to the gonads in both the triploids and diploids.

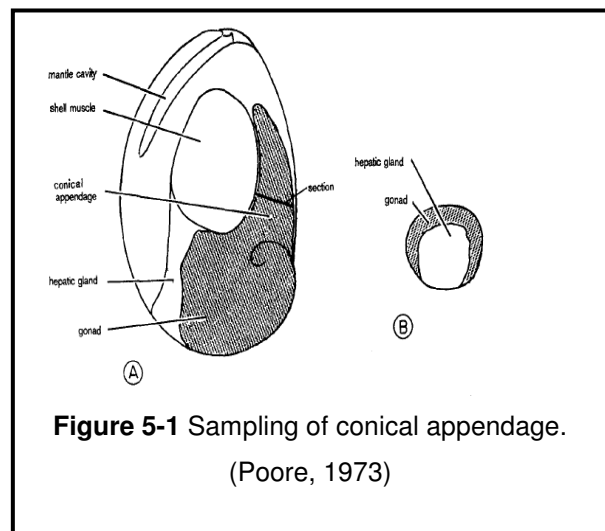
In a study by Dunstan *et al.* (2007) on greenlip abalone (*Haliotis laevis*), diploid abalone between the age of 13 to 48 months were consistently 11 - 15% longer than their triploid siblings. Triploid abalone in their study yielded 30% higher meat weight in the spring-summer maturation period than diploid abalone of similar shell length. In the same study, Dunstan *et al.* (2007) reported that between these maturation periods the diploids had compensatory growth and had meat weights comparable to that of the triploids. They concluded that during the maturation periods triploid abalone harvested for canning was cost effective.

There may also be a ploidy and sex interaction in yield during the reproductive season in triploid individuals since triploidy affects the two sexes differently (Perruzi, *et al.*, 2004). Dunstan *et al.* (2007) however found no variation in fatty acid composition in the meat of 48-month old greenlip abalone (*H. laevisgata*) regardless of ploidy, maturity or sex.

The lower meat yield in diploids compared to triploids suggests a reduced gametogenesis in triploid abalone. This has a commercial implication on FCR during natural spawning periods in diploid individuals with energy diverted to reproductive maturation in diploid genotypes resulting in an increased FCR, which is not the case in triploids.

### 5.1.2 Effect of triploidy on gonadal development, measured as gonadosomatic index (GSI)

Gonadal development in this study was measured as gonadosomatic index (GSI). GSI is calculated using the conical appendage, consisting of the conical hepatic gland covered by a gonad sheath (Poore, 1973). It is determined as the percentage of the total area of gonadosomatic tissue (gonad sheath) over the total area of gonadosomatic tissue and the hepatic gland at a set one third cross-section of the conical tip and is illustrated in Figure 5.1. This method is similar to the one used by Ino and Harada (1961) (Japanese Haliotids), Boolootian *et al.* (1962) (Californian Haliotids), and Newman (1967) (South African Haliotids). This gonad index is useful in experimental studies since it is independent of size in trial animals.



Triploidy does not always produce complete sterility in shellfish, but rather a decreased gonadal development (Piferrer, *et al.*, 2009). However, sterility is associated with decreased gonadal size in molluscs (Liu, *et al.*, 2009). Examples of decreased

gametogenesis in triploid bivalves include *Argopecten irradians* (Tabarini, 1984), *Chlamys nobilis* (Wada, *et al.*, 1989), *Argopecten ventricosus* (Ruiz-Verdugo, *et al.*, 2000), *Mya arenaria* (Allen, *et al.*, 1986) and *Haliotis rubra* (Liu, *et al.*, 2009).

Functional gametes as well as spawning have been found in triploid shellfish. These include the Japanese pearl oyster, *Pinctada fucatamartensii* (Wada, *et al.*, 1989), the Pacific oyster, *Crassostrea gigas* (Allen and Downing, 1986) and Pacific abalone, *Haliotis discus hannai* (Li, *et al.*, 2004) to name only a few. It is important to note that in the above examples functional gametes were produced at low rates. This will not prevent but rather limit their spawning capacity (Liu, *et al.*, 2009).

As previously mentioned, complete sterility or even only a limited reproduction capacity is desirable in the commercial culture of abalone in South Africa. This is true because over 60% of all exports are live and random spawning during transport affects product quality adversely. Even in the absence of complete sterility, reduced gonadosomatic indices will have positive implications for important production parameters in triploid variants of the South African abalone, *H. midae*, such as processing yield and unwanted random natural spawning.

## **5.2 Materials and Methods**

### **5.2.1 Materials**

The animals used in this trial were selected from the same three distinct progeny groups produced in 2006 at quarterly intervals in accordance with a procedure described by Schoonbee (2008) as mentioned previously in section 3.2.1 and 4.2.1.

### **5.2.2 Sampling**

Samples were taken during three stages outside of normal spawning season and then again at three stages during the normal spawning season (spring or September in South Africa). Twelve diploid and twelve triploid individuals were randomly selected at three different ages (i.e. 56, 59 and 62 months) during the May part of the trial. Twelve individuals per ploidy, again for three distinct ages, were selected during the natural

spawning period (September) of the trial. A summary of the experimental layout can be seen in Table 5.1 below.

This method of sampling allows for the calculation of yield percentage and GSI over time as well as distinguishing between these percentages during spawning periods. For yield analysis and GSI, the animals had to be sacrificed through destructive sampling.

**Table 5-1** The experimental layout for canning yield and GSI trial performed during Phase 2 of a comparative growth analysis between triploid and diploid variants of *Haliotis midae*.

	May			September		
Age	56 months	59 months	62 months	60 months	63 months	66 months
Triploid	12	12	12	12	12	12
Diploid	12	12	12	12	12	12

Shell length was measured to the nearest 0.1 mm using Vernier Callipers and weight (at various stages of processing) to 0.1 g with a digital scale.

Gonadosomatic index (GSI) was defined as:

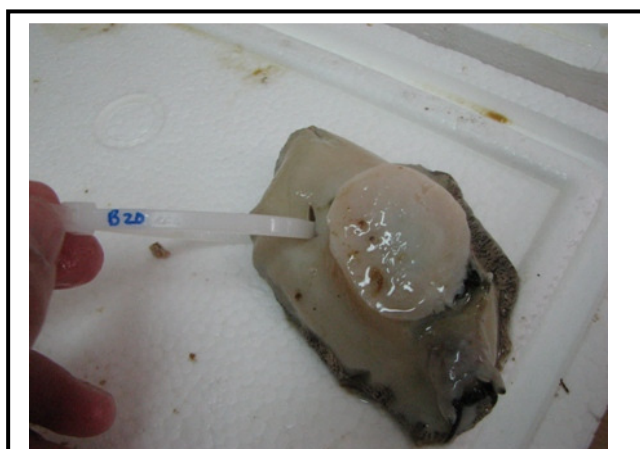
$$GSI = \frac{\text{Gonadal area}}{\text{Total area}} \times 100\%$$

Final yield was defined as:

$$\text{Yield percentage} = \frac{\text{Final product weight}}{\text{Starting live weight}} \times 100\%$$

### 5.2.3 Processing Yield

The selected animals were purged overnight in mesh storage bags before processing began the next morning. The abalone were transported from Danger Point near Gansbaai to the canning facility, Walker Bay Cannery, in Hermanus.



**Figure 5-2** Tagging of abalone for canning analysis.



On arrival, each individual was weighed and a Cable Tie™ with a corresponding number was assigned. The Cable Tie™ allowed the marking and tracking of each individual abalone through the process of canning. The animals were then shucked and measurements of the shell, the body and soft tissue (intestines) were recorded. The body was tagged with the Cable Tie™ by cutting a hole through the foot muscle using a scalpel and attaching the Cable Tie™ through the hole as illustrated in Figure 5.2.

After shucking and subsequent measurements, the bodies were placed in salt brine overnight according to standard processing procedures. The following morning the product was scrubbed clean



and the head and epipodia were trimmed off, after which each individual had its weight recorded again. The product was placed in cans with fresh water and sterilized under steam for several minutes as shown in Figures 5.3 and 5.4. All the cans were then placed in a steam oven and cooked for 55 minutes at 121 °C (Figure 5.5).

When the cans had cooled after the heat retort, each individual was again weighed for the final time and processing yield calculated.



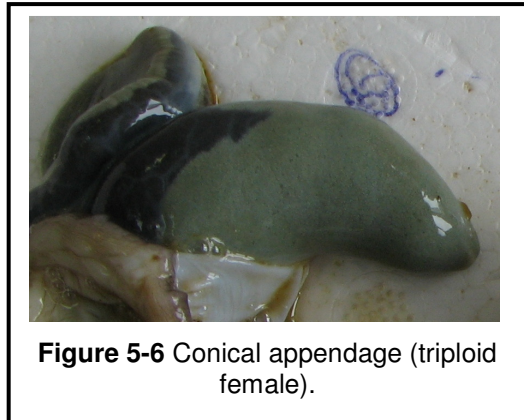
**Figure 5-4** Sealed cans are sterilized in a steam machine.



**Figure 5-5** Cans placed in a steam cooker for a heat retort.

### 5.2.4 Gonadosomatic index

Six out of every twelve individuals per ploidy were randomly selected from each of the age groups assigned for processing yield. GSI analysis required only the conical appendage (Figure 5.6) and these were taken from the soft tissue after shucking and weighing in the processing yield analysis.

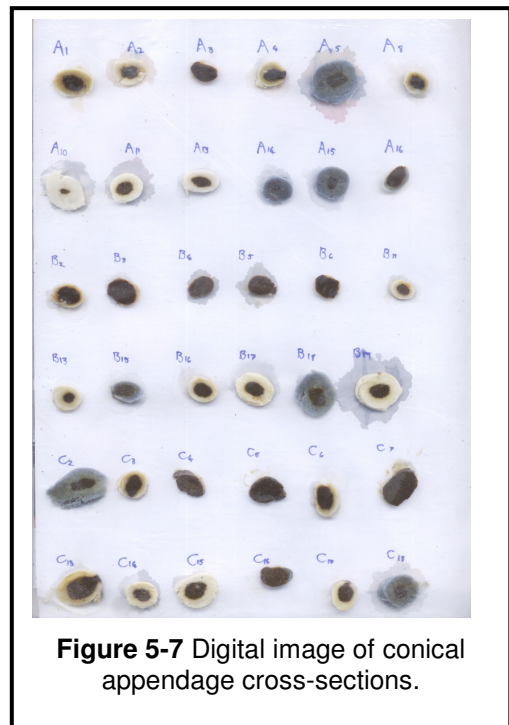


The complete conical appendage was preserved in saline formaldehyde for 48 hours. After the entire appendage had hardened, it was sectioned at a point one third the distance from the shell apex to its tip. These cross-sections

were then placed on a transparent plastic and scanned into a computer using a digital scanner (Figure 5.7).

A computer program, ImageJ, used for the mapping of cancer tumours was used to determine the exposed surface areas. For each individual the gonadal tissue surface area was determined as well as the surface area of the entire cross-section. The values obtained were used to calculate each individual GSI from the formula mentioned in section 5.2.2.

Samples in each instance were randomly sampled allowing three female and three male measurements per ploidy for each age group. The GSI in relation to age as well as to spawning season was then determined.



### **5.2.5 Gonadal development**

The gonads of *H. midae* adults show a strong colour dimorphism and allow for the visual distinction between mature diploid abalone. The male abalone has a cream to off-white coloured gonad, while the female gonad is blue or greenish in colour. The gonads of all individuals in the destructive part of the trial were visually assessed and differences noted.

## **5.3 Results and Discussion**

The main objective of this study was to assess if there were differences between diploid and triploid genotypes of the abalone, *H. midae*, regarding the production parameters yield and GSI during maturation.

Secondary objectives were to establish the relationship of these production parameters over time as well as during spawning season.

### **5.3.1 Data analysis**

Weight and surface area measurements were sampled at six age intervals during two natural seasons, which resulted in data for the analysis of yield and GSI percentage.

For each one of the two production parameters measured the experimental layout consisted of a Random Block Design. The experimental layout consisted of two Blocks, namely seasons and two Treatments (triploid and diploid).

The data was then analyzed in a progressive manner to:

- a. Assess the effect of ploidy on the average processing yield percentage by means of a covariate analysis.
- b. Assess the effect of ploidy by means of regression analyses for yield percentage on age.
- c. Assess the effect of ploidy on the average GSI by means of a covariate analysis.
- d. Assess the effect of ploidy by means of regression analyses for GSI on age.

- e. Describe the GSI-Yield relationship for triploid and diploid groups over a period of 56 to 66 months.

### 5.3.1.1 Assessment of the effect of ploidy on the average processing yield percentage

The age-adjusted means were calculated over Blocks to ignore seasonal effects. Ignoring seasonal influences allows for the observed differences in yield percentages to be ascribed to the differences between triploid and diploid treatments, i.e. the effect of the induction. The age-adjusted means are presented in Table 5.2 below.

**Table 5-2** The age-adjusted mean yield percentages of triploid and diploid abalone, *Haliotis midae*, over Blocks.

Parameter	Treatment	Adjusted Mean	F-value	p-value
Yield	Triploid	28.18%	20.817	<0.001
	Diploid	25.18%		

The results gave an indication of significant differences between the yield percentages of the triploid and diploid abalone assessed over Blocks; thus ignoring the seasonal effects. The differences between triploid and diploid yield percentages (3.01%) have considerable economic implications when considering canned processing. The triploid specimens showed an average total improvement over the diploid individuals of 10.6%, which will have significant economic implications considering the high tonnage processed commercially.

It is well known that in *H. midae*, animals have a natural spawning period in the year. This is in the South African spring, during September and October. During the spawning period, the gonads become expanded and more energy is allocated to gametogenesis. The practical implication on yield percentage is that there is an increased amount of soft tissue when compared to the rest of the year. It was therefore thought important to assess differences in yield percentages during different seasons.

The age-adjusted means were calculated between Blocks to incorporate the seasonal effect and determine if there was any interaction between spawning season and the treatment. The age-adjusted means are presented in Table 5.3 below.

**Table 5-3** The age-adjusted mean yield percentages of triploid and diploid abalone, *Haliotis midae*, between Blocks with the results of a pair-wise T test to indicate the similarity of the mean yield percentages for seasons. (For p-values > 0.05, the means were considered as similar and for p < 0.05 the means were considered as statistically different from each other.)

Parameter	Season	Adjusted Mean	T test	t-value	p-value
Yield (triploid)	May	28.33%	a	1.28	0.2047
	September	28.15%	a		
Yield (diploid)	May	26.95%	b	2.36	0.0219
	September	23.80%	c		

The above results indicate that the age-adjusted means for triploid yield do not differ significantly from each other. This suggests that, based on the above data, not only do triploid individuals have a higher yield ignoring seasons, between Blocks triploid individuals do not have the drop off in yield as the spawning season approaches. The yield remained statistically unchanged between the two seasons. The diploids however showed the opposite. There was a statistically significant difference between diploid yield percentages during the two spawning seasons for a 95% confidence interval. This shows that there was the expected decreased yield within the spawning season for diploid variants.

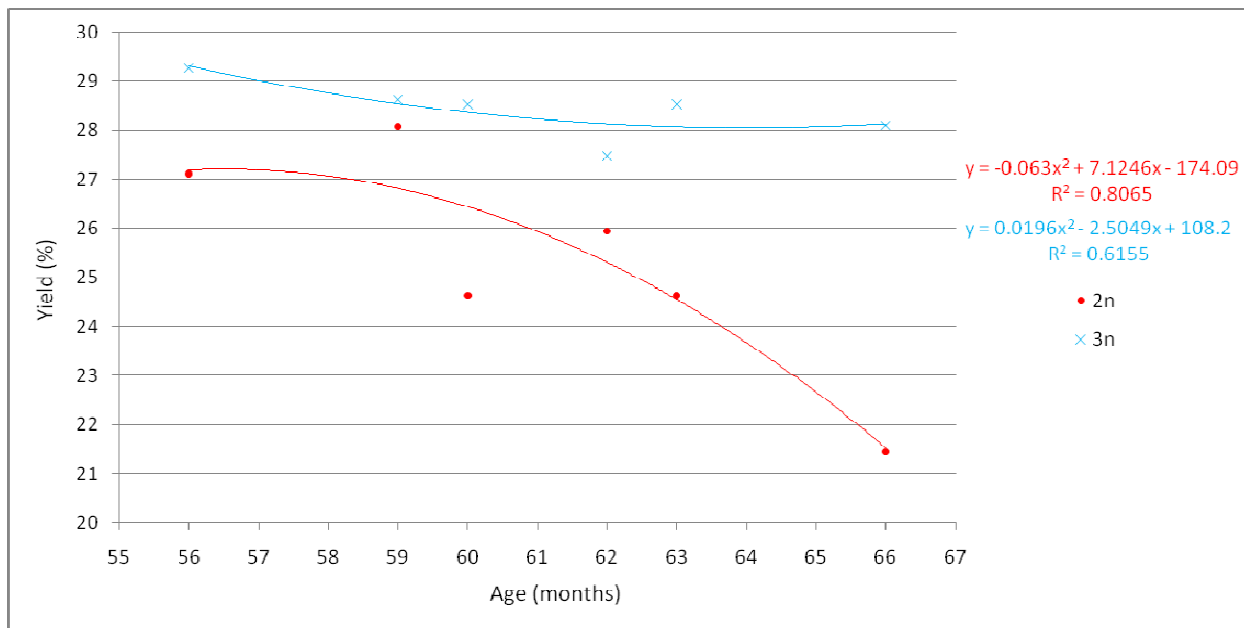
### 5.3.1.2 Assessment of the effect of ploidy on the basis of the regressions of yield percentage on age

The relationship between yield percentage and age has commercial implications in the culture of *H. midae* in South Africa. Abalone reaches sexual maturity during the production period and the increased energy expenditure into gametogenesis results in a decreased yield percentage. This can be simplified by saying that more energy from

feed is needed to produce the same final processed product in an individual that is sexually mature compared to a juvenile.

Gametogenesis in Haliotids should be positively correlated to size and an increase in age with accompanying growth will result in a decreased yield percentage when regarding that soft tissue (intestine and gonads) does not contribute towards the final product.

The relationship between yield percentage and age of abalone, based on the regression of the yield percentages and ages of diploid and triploid abalone is presented in Figure 5.8.



**Figure 5-8** The relationship between mean yield percentage and age in triploid (blue crosses) and diploid (solid red circles) *Haliotis midae*. The respective equations and R-squared values are displayed on the right.

The relationship of yield percentage in triploid and diploid abalone over time is described by the quadratic equations:

Diploid equation:  $Y = -0.063x^2 + 7.1246x - 174.09$

Triploid equation:  $Y = 0.0196x^2 - 2.5049x + 108.2$

where  $Y$  = yield measured as percentage and  $X$  = age (months).

A quadratic regression had the most appropriate fit to the data and the R-squared values, 0.807 and 0.616 for diploid and triploid, gave an indication of this. The effect of triploidy on yield percentage over time can easily be observed from the above graphs. It is important to note the decrease (the so-called “drop off”) in yield with the increase in age in the diploid individuals.

### 5.3.1.3 Assessment of the effect of ploidy on the average GSI percentage

Triploidy can influence *Haliotids* by inducing sterility. Triploidy induced sterility does however not necessarily mean that there will be no gametogenesis present in triploid individuals. More likely, a reduced or retarded gametogenesis in triploid variants will occur, that confers the triploid growth advantage, if any. Measuring the gonadosomatic indices in triploid and diploid variants over time enables to the determination of differences in GSI percentages in triploid and diploid *H. midae*).

The age-adjusted means were calculated over Blocks to ignore seasonal effects. Ignoring seasonal influences allows for the observed differences in GSI percentages to be ascribed to the differences between triploid and diploid treatments, i.e. the effect of the treatments. The age-adjusted means are presented in Table 5.4.

**Table 5-4** The age-adjusted mean GSI percentages of triploid and diploid abalone, *Haliotis midae*, over Blocks.

Parameter	Treatment	Adjusted Mean	F-value	p-value
GSI	Triploid	49.26%	34.5374	<0.001
	Diploid	77.68%		

The results gave an indication of significant differences between the GSI percentages of the triploid and diploid abalone assessed over Blocks; thus ignoring the seasonal influences. The differences between triploid and diploid GSI percentages (28.42%) have considerable economic and practical implications when considering canned processing and live export. Spawning that occurs during stressful periods such as transport,

negatively impact the quality and thus the value of the abalone. A reduced gonadosomatic activity will ease management in that feed conversion ratios can be calculated without the loss in weight through random spawning. There is an increased feed intake with a loss in mass during spawning. If triploid abalone does not show increased growth, but have reduced GSI's, then it is possible that they have reduced feed intake. Even if there were no increased growth, a reduced energy budget would still be beneficial to the commercial industry. Feed costs make up a substantial part of the cost relating to the expensive grow-out of abalone.

#### **5.3.1.4 Assessment of the effect of ploidy on the basis of the regressions of GSI percentage on age**

Similarly to the relationship of yield percentage and age, as mentioned above in section 5.3.1.2, the relationship between gonadosomatic index percentage and age has important commercial implications. There is not only a financial consequence associated with gonadosomatic development and maturation, but a practical managerial inference as well.

Energy is utilized for gametogenesis as abalone mature and it results in less energy from feed being utilized for somatic growth. Simply stated, this equates into money spent on unwanted (not marketable) growth and development. The live transport and canning of abalone is also negatively affected by mature gonads. In live transport, the stress regularly induces spontaneous spawning that reduces the product quality as well as the live weight. Gonads are not included in the final canned product and any growth achieved in gametogenesis is seen as wasted energy.

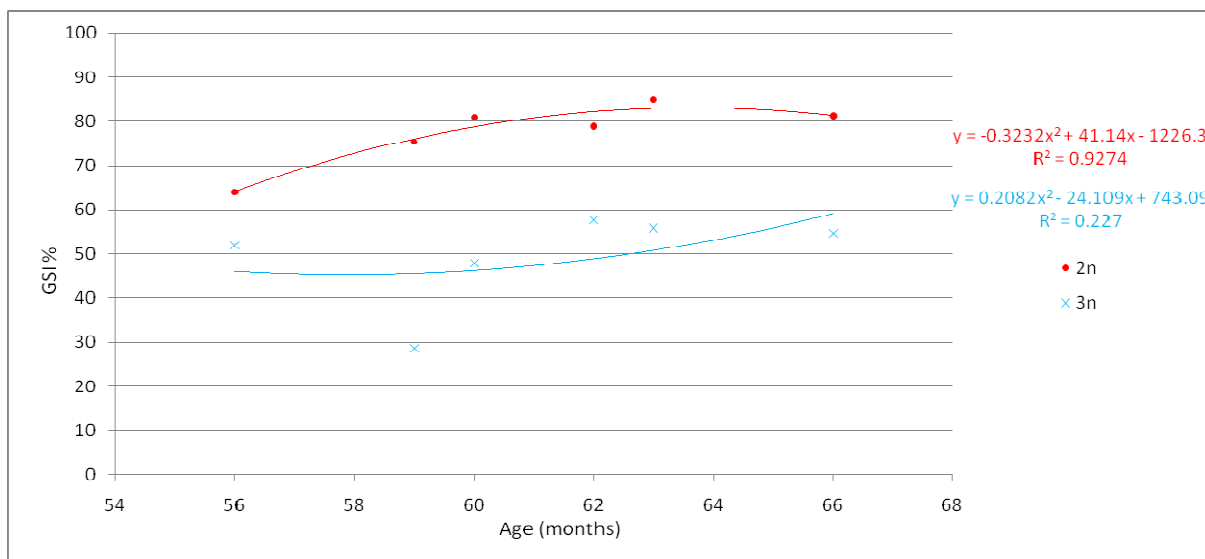
In the production of an on-growing unit, feed conversion ratio (FCR) becomes an important managerial tool to monitor and coordinate the farming system. The FCR is generally calculated as the increase in weight of the total biological mass (the abalone) divided by the total weight of feed given to achieve that increase in mass. When the abalone have mature gonads and a random spawning event occurs, a large proportion of biological mass is lost with no decreased feed consumption. Practically this makes



calculating and managing FCR's difficult and farm managers regularly refer to a drop-off in FCR during the natural spawning periods.

As mentioned in section 5.3.1.2, gametogenesis should be positively correlated to size in Haliotids and an increase in age with accompanying growth will result in an increased GSI percentage.

The relationship between GSI percentage and age of abalone, based on the regression of the GSI percentages and ages of diploid and triploid abalone is presented in Figure 5.9.



**Figure 5-9** The relationship between mean GSI percentage and age in triploid (blue crosses) and diploid (solid red dots) *Haliotis midae*. The respective equations and R-squared values are displayed on the right

The relationship of GSI percentage in triploid and diploid abalone over time is described by the quadratic equations:

$$\text{Diploid equation: } Y = -0.3232x^2 + 41.14x - 1226.3$$

$$\text{Triploid equation: } Y = 0.2082x^2 - 24.109x + 743.09$$

where Y = GSI measured as percentage and X = age (months).

A quadratic regression again had the most appropriate fit to the data. The R-squared value ( $R^2 = 0.227$ ) of the triploid equation is low but regardless gives a good indication of the effect of triploidy on gonadosomatic index percentage over time.

Application of the above equations to predict the respective GSI percentage of abalone at 56 and 66 months provided the following results, which confirmed the significance in difference in GSI percentage increase between triploids and diploids:

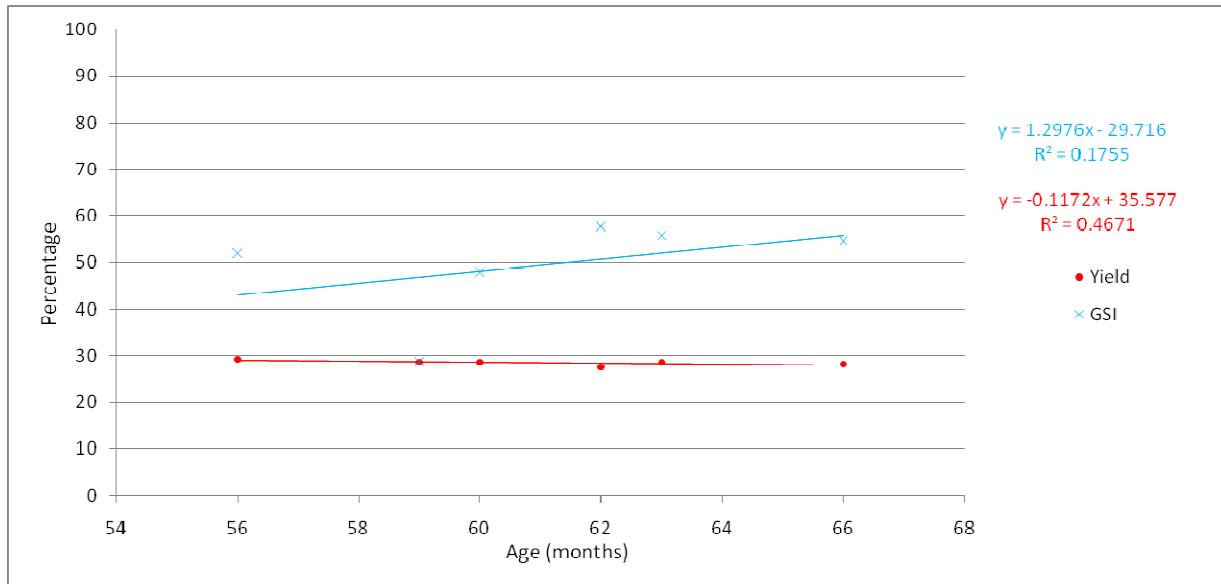
Average GSI percentage at 56 months: Triploid	= 45.90%
Diploid	= 63.98%
Average GSI percentage at 66 months: Triploid	= 58.82%
Diploid	= 81.08 %

Both showed an expected increase in GSI percentage with an increase in age; the triploid individuals however showed a much lower average during this trial period. The triploid individuals indicated evidence of retarded gonadal development.

#### **5.3.1.5 Correlation between GSI percentage and yield percentage over a period of 56 to 66 months**

Considering that there is a clear correlation between age and yield and GSI percentages, one would expect a correlation between yield percentage and GSI. There is indeed a negative correlation between yield percentage and GSI. With an increase in GSI (measured as a percentage), or rather an increase in soft tissue percentage, there will be a reduced yield percentage when considering muscle tissue.

A strong negative correlation between these two production parameters is expected in the diploid state but it is the purpose of this study to investigate if this is true for triploid *Haliotis midae*. This relationship can be seen in Figure 5.10 and 5.11.



**Figure 5-10** Relationship between GSI percentage (blue crosses) and Yield percentage (solid red dots) over time for triploid *Haliotis midae*. The respective equations and R-squared values are displayed on the right.

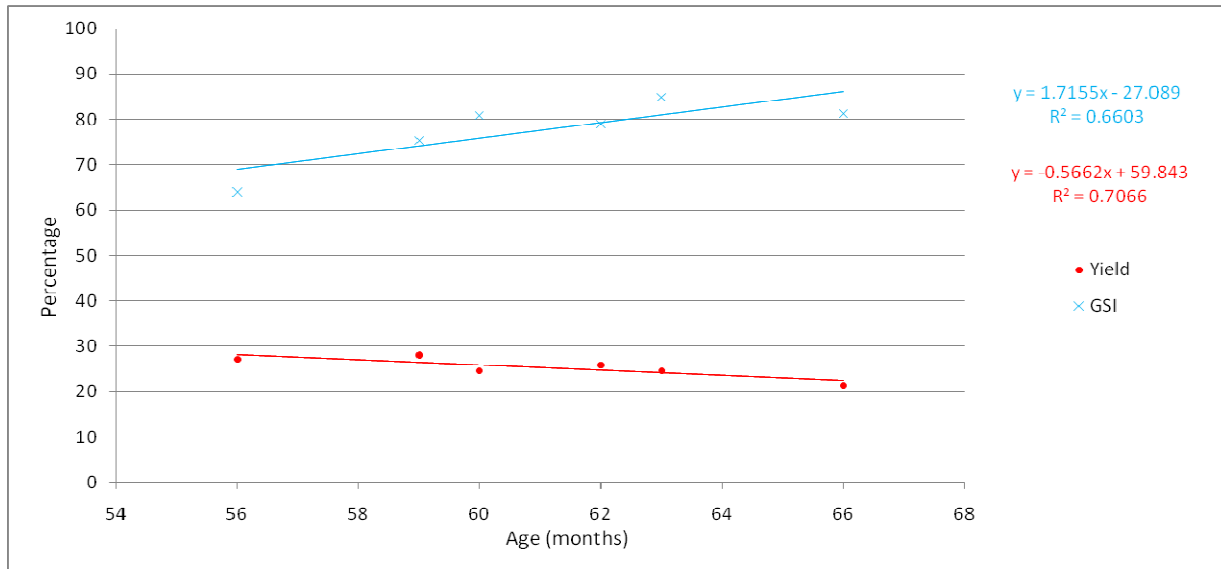
The relationship between Yield and GSI percentage over age (months) for triploid *H. midae* is given by the equations:

GSI percentage linear equation:  $Y = 1.2976x - 29.716$

Yield percentage linear equation:  $Y = -0.1172x + 35.577$

where Y = percentage and X = age in months.

The correlation coefficient = - 0.35



**Figure 5-11** Relationship between GSI percentage (blue crosses) and Yield percentage (solid red dots) over time for diploid *Haliotis midae*. The respective equations and R-squared values are displayed on the right

The relationship between Yield and GSI percentage over time for diploid *H. midae* is given by the equations:

GSI percentage linear equation:  $Y = 1.7155x - 27.089$

Yield percentage linear equation:  $Y = -0.5662x + 59.843$

where Y = percentage and X = age in months.

The correlation coefficient = - 0.60

The resultant correlations for triploid and diploid *H. midae* confirm that negative correlation between GSI and yield percentage was present in this study. The diploid individuals showed a much stronger negative correlation compared to the triploid individuals. This clear difference in correlation coefficients is indicative of a possible influence of triploidy on normal gonadal maturation in mature *H. midae*.

## 5.4 Summary

The effect of triploidy on the processing yield and gonadosomatic index of *H. midae* was determined using regression and covariate analysis. The GSI to Yield relationship was also described and the respective correlations calculated.

When considering that the yield percentage of triploid individuals remained stable with an increase in age and with seasonal variation when compared to their diploid counterparts, it becomes easy to assume that the effect of the triploid treatment reduces gonadal development in *H. midae*.

The reduction in gonadal maturation results in a higher yield percentage in triploid individuals compared to diploid individuals. Not only is there a higher average yield in triploid genotypes, the percentages remain more constant with an increase in age and there is no seasonal variation as observed in diploid genotypes. This further supports the theory that triploidy as a treatment induces sterility, or at least in this study, reduces gonadal development severely.

The benefits that this reduction in gonadosomatic activity holds for the commercial farming of *H. midae* justify the slight loss of growth performance that the induction of triploidy in this abalone species brings. The financial gain through canning and improved product quality during live exports surpasses the reduction in growth and justifies the commercial application of triploid induction in *H. midae*.

## 5.5 References

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## 6. Conclusion

The main objective of this study was to conclude the comparative assessment of the growth rate of triploid and diploid genotypes of the abalone, *Haliotis midae*, to an age of sexual maturity and reproduction as recommended by Schoonbee (2008). An additional objective was to assess other important production traits that could be affected by the ploidy status, including higher percentage yield, improved meat quality, biosecurity advantages through sterility and improved management options also recommended by Schoonbee (2008).

Significant differences were detected in growth rate, with the diploid genotypes displaying superior wet weight (+5.13 g) and shell length (+1.99 mm) at 62 months of age, which equates to about one month of on-farm production. Triploid individuals showed a 10.6% higher yield than diploids during the canning trial with mean GSI for triploids 28.42% lower than that of the diploid variants.

The significantly lower GSI of the triploids was also related to an inability of this genotype to spawn at an age well past sexual maturity (92 months), compared to the diploid siblings that displayed normal spawning patterns. The prevention of spawning has beneficial impacts on production through improved condition, meat quality and yield, as well as during live export due to improved water quality, reduced stress, reduced weight loss and improved condition.

A method has also been developed for the rapid and accurate validation of ploidy status in *H. midae* through the use of species-specific microsatellite markers.

The application of the technology to induce triploidy in commercial production of *H. midae* is therefore recommended on the basis of:

- a 10.6% improvement in yield,
- improvement in general condition and
- the prevention of uncontrolled spawning events during the late production phase and live transport,

taking into account the 1.6% reduction in overall growth.



Research in this field of study can be improved through:

- the implementation of a more reliable tagging method,
- assessment and improvement of the survival of triploids during larval and settlement stage of development,
- comparison of the metabolic requirements of triploid and diploid genotypes in terms of oxygen, energy and feed conversion,
- comparison of the stress tolerance of triploid and diploid genotypes,
- the measurement of gametogenesis in triploid abalone and their use for biological containment and,
- the further development of the microsatellite markers to improve the accuracy of the technique for verification of the ploidy status of genotypes.

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