# The evaluation of biological, mechanical and chemical methods to contain South African abalone species (*Haliotis midae*)

by
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# Declaration

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## Abstract

*Haliotis midae* (HM) is one of five indigenous abalone species occurring along the coast of South Africa, and is the only species commercially cultivated in open flow-through systems. Commercial production of HM contributes the most to income generated from aquaculture species in South Africa. Exports in 2011 totalled 1036 ton, which amounts to an income of ZAR 369 million (40 million USD). Commercial production of HM is characterized by a long interval (i.e. up to five years) to harvest. Genetic modification of HM to improve growth rate in HM can potentially decrease the interval from hatch to harvest, which will contribute to optimising the cost-efficient production of HM. However, a major concern in the production of genetically modified (GM) abalone is their escape from production systems, and the potential impact of the GM animals on the natural stocks in the surrounding environment. The implementation of containment methods to prevent the escape from GM abalone from production systems will assist in the management of the potential ecological risks escaped GM abalone may pose. Currently no containment strategies exist for the South African abalone industry, which limits the use of transgenic biotechnologies to optimise the cost-efficient production of HM. The study therefore investigated the potential of biological methods (triploid induction), mechanical methods (polypropylene filter bags and obstructive materials), and chemical methods (sodium hypochlorite and chlorine dioxide) to contain transgenic HM in open flow-through systems.

Histological evaluation of gonad tissue samples of 16 triploid male and 15 triploid female HM indicated that triploid induction was unsuccessful in arresting gonad differentiation, development, and function. A combination of immature and mature gametes was observed on both triploid male and female gonads. The fertilizing potential of biopsied sperm obtained from triploid and induced diploid male HM was assessed in fertilization trials using ova obtained from diploid HM. No larvae were observed from the fertilization treatments of triploid male biopsied sperm, however, abnormal larvae (0.01% fertilization) were observed when induced diploid sperm were used to fertilize diploid ova.

Polypropylene filter bags (100 µm) were not effective in containing male and female gametes in the broodstock section of the abalone culture system used in this study, and future studies should address specialized methods of containment of HM gametes. Polypropylene filters (100 µm), resulted in effective containment of larvae and settlement larvae, and in this study was not a more effective method of containment than the current on-farm larvae containment protocol. The three obstructive materials were effective in containing HM spat, and did not differ in their ability as containment methods.

Sodium hypochlorite and Biox™, chlorine-based chemicals, were effective in containing the five life stages of HM, i.e. sperm, ova, larvae, settlement larvae and spat. The respective life stages were exposed to varying concentrations of the respective chemicals (i.e. 10 ppm, 50 ppm and 100 ppm) over five exposure times (i.e. 4, 6, 8, 10 and 12 minutes). Sodium hypochlorite and Biox™, at the different concentrations and exposure

times, resulted in mortalities in all five HM life stages, with higher incidences recorded at the higher concentrations evaluated.

Future research on the effect of triploid induction on the endocrine system, and how effective gonad maturation and function is suppressed, needs to be conducted. The aspects of mosaics occurring in induced diploid animals warrant further investigation with a larger sample size to verify the findings of this study. The production of true sterile individuals would improve the results to use of GM animals as a method of biological containment. Incorporation of mechanical containment methods need to be kept in mind when abalone production systems are designed. The use of chemical containment methods warrants additional studies on the practical administration and the economic feasibility of the chemical into the water supply of open flow-through abalone culture systems.

# Uitreksel

*Haliotis midae* (HM) is een van vyf inheemse perlemoen spesies wat langs die kus van Suid-Afrika voorkom en is die enigste spesie wat kommersieel in oop deurvloeistelsels geproduseer word. Kommersiële produksie van HM dra die meeste tot kommersiële akwakultuur produksie by, met uitvoere van 1036 ton in 2011, wat gelykstaande aan ‘n inkomste van ZAR 369 miljoen (40 miljoen VSA dollar). Kommersiële produksie van HM word gekenmerk deur 'n lang interval (d.i. tot 5 jaar) vanaf uitbroei tot oes. Genetiese modifisering van HM om die groeitempo te verbeter kan potensieel die interval tot slagmassa verkort, wat potensieel tot die optimalisering van die kostedoeltreffende produksie van HM kan bydra. 'n Groot bron van kommer met die produksie van geneties gemodifiseerde (GM) perlemoen is hul ontsnapping uit produksiestelsels en die potensiële impak van die GM diere op die natuurlike perlemoenpopulasies in die omliggende omgewing. Die implementering van beheermetodes om die ontsnapping van GM perlemoen uit produksiestelsels te voorkom sal die bestuur van die potensiële ekologiese risiko's wat GM perlemoen mag inhou, vergemaklik. Tans bestaan daar geen inperking strategieë vir GM diere vir die Suid-Afrikaanse perlemoenbedryf nie, wat die gebruik van transgeniese biotegnologieë vir die kostedoeltreffende produksie van HM beperk. Die studie het dus die potensiaal van biologiese metodes (triploïed induksie), meganiese metodes (polipropileen filters en obstruktiewe materiaal) en chemiese metodes (natriumhipochloriet en Biox™) om as inperk strategieë vir die bestuur van transgeniese HM in oop deurvloei produksiesisteme gebruik te word, ondersoek.

Histologiese evaluering van gonadeweefsel monsters van 16 manlike en 15 vroulike triploïed HM het aangedui dat triploïed induksie onsuksesvol was om gonade differensiasie, ontwikkeling, en funksie te onderdruk. 'n Kombinasie van onvolwasse en volwasse gamete is in gonades van beide manlike en vroulike triploïed HM waargeneem. Die bevrugtingsvermoë van sperme wat versamel is met behulp van ’n biopsie metode van triploïed en geïnduseerde diploïed HM is geëvalueer in bevrugtingsproewe met ova wat van diploïed HM versamel is. Geen larwes is waargeneem met die gebruik van die triploïed sperme nie, maar abnormale larwes (0.01% bevrugting) het wel voorgekom met die evaluering van sperme versamel van geïnduseerde diploïed HM. Die voorkoms van mosaïek diere (d.i. ’n kombinasie van beide diploïed en triploïed selle in een individu) wat ontstaan a.g.v. onvolledige triploïed induksie noodsaak verdere navorsing met ’n groter monster diere om die vermoë van triploïed induksie om HM steriel te maak, te verifieer.

Polipropileen filters (100 µm) was nie effektief om beide manlike en vroulike gamete wat in die teelafdeling in die perlemoenkultuursisteem in hierdie studie geproduseer is, vas te vang nie. Toekomstige studies moet gespesialiseerde metodes wat insluiting van HM gamete moontlik sal maak, ondersoek. Polipropileenfilters (100 µm) was effektief met die inkamping van larwes en vestigingslarwes, met die filters wat nie meer doeltreffend as die bestaande inkampingsmetodes in die produksiesisteem was nie. Die drie obstruktiewe materiale was doeltreffend ten opsigte van die inkamping van onvolwasse perlemoen individue, met die 3

materiale wat ewe doeltreffend in hulle vermoë was om die onvolwasse HM se ontsnapping uit die sisteem te voorkom.

Natriumhipochloriet en Biox™, chloor-gebaseerde chemikalieë, was effektief om die onderskeie 5 lewenstadiums, d.i. HM sperme en ova, larwes, vestigingslarwes en onvolwasse HM se voorkoms tot hulle onderskeie afdelings binne die produksiesisteem te beperk. Die onderskeie lewensstadiums is blootgestel aan verskillende konsentrasies van die onderskeie chemikalieë (d.i. 10 dpm, 50 dpm en 100 dpm) oor vyf tydsintervalle (d.i. 4, 6, 8, 10 en 12 minute). Natriumhipochloriet en Biox™ het ’n letale effek op al vier lewensstadia gehad, met hoër sterftes wat met hoër konsentrasies aangeteken is.

Toekomstige navorsing oor die uitwerking van triploïed induksie op die endokriene stelsel en hoe doeltreffend die rypwording en funksie van die gonades onderdruk word, moet aandag geniet. Die voorkoms van mosaïek diere regverdig ook spesifieke aandag in toekomstige navorsing. Die produksie van ware steriele individue sou die gebruik van GM perlemoen as 'n metode van biologiese inkamping moontlik maak. Inlywing van meganiese inkampingmetodes moet in gedagte gehou word wanneer perlemoen produksiestelsels ontwerp word. Die gebruik van chemiese inkampingmetodes benodig verdere studies om te verseker dat die chemiese middels so bekostigbaar en prakties as moontlik in water van 'n oop deurvloei perlemoen produksiestelsel toegedien kan word.

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# Chapter 1

# General Introduction

Internationally the aquaculture industry is the fastest growing industry in the animal production sector, growing at an average annual rate of 6.6% per year since the 1970s (FAO, 2010). Between 1961 and 1999, the global demand for fish protein sources increased from 28 to 96.3 million metric tons, and to a further 133 million metric tons from 1999 to 2001 (FAO, 2010).

The high demand for abalone products from the East (Tarr, 1989; Britz *et al.,* 2009) led to the establishment of the South African abalone industry in 1949, exporting abalone harvested from the wild. Commercial abalone farming started in 1990, and has since developed into the most valuable sector within the South African aquaculture industry. Restrictions on the harvesting of wild stock has led to an increase in cultivation of abalone (Troell *et al.,* 2006), with 12 commercial farms located along the coastline of South Africa in 2012 (DAFF, 2012)

As the global demand for marine-based food is increasing, aquaculture and transgenic technologies are being investigated to alleviate the increased pressure and consequent excessive over utilization. The use of biotechnology, although still at an early stage in the research, could potentially contribute towards the alleviation of over-exploitation (Devlin *et al.,* 2006; Wong and Van Eenennaam, 2008). The application of such biotechnologies are based on enhancing desired traits such as increased growth and feed conversion rates, which are two of the most commercially important traits in aquatic species production (Devlin *et al.,* 2006; Rasmussen and Morrissey, 2007; Wong and Van Eenennaam, 2008).

Genetically modified organisms (GMOs) are organisms of which the genetic make-up has been altered to improve specific production traits (Morse, 1977; Wong and Van Eenennaam, 2008). Genetically modified organisms can include plants, animals or microorganisms (Whitman, 2000), and form part of various industries such as the wine industry, aquaculture, crops, and pharmaceuticals. Genetically modified organisms varieties are developed to improve the efficiency of production (Hallerman and Kapuscinski, 1995; Beardmore and Porter, 2003), animal health product quality, to mitigate the environmental impact of food and animal production, and to produce biopharmaceuticals and industrial biochemical products (Kochlar and Evans, 2007). Transgenic modification of fish resulted in an improvement in several economically and ecologically important traits such as growth rate, feed conversion efficiency, disease resistance, cold tolerance, increased carcass yield, increased protein levels and reduced fat in their meat (Beardmore and Porter, 2003; Devlin *et al.,* 2006; Hu *et al*., 2007; Wong and Van Eenennaam, 2008; Dunham, 2009). The use of GMOs is envisaged to alleviate the decline of the natural stocks, as well as increase supply through enhancing the growth rate of aquatic animals.

A major concern of the production of GMOs centres around the potential effect of transgenic escapees on wild stocks (Beardmore and Porter, 2003). The development of sterile genotypes in order to reduce the potential impact of genetically modified escapees on natural stocks currently form part of the focus of several research programs (Beardmore and Porter, 2003). Often genetically modified animals will have a compromised reproductive ability, which; even though the genetically modified animal could potentially have an increased mating success, their offspring could experience a decrease in viability, which can potentially result in the extinction of both the wild and transgenic populations (Muir and Howard, 1999).

Containment strategies would potentially minimize the negative impact on the environment that is associated with the use of GMOs (Devlin *et al.,* 2006). Containment strategies can involve containment by sterility, biological containment, containment by geographical location, chemical containment, and physical containment. In order to ensure the safe application of transgenic aquaculture, the effective implementation of physical, chemical and/or biological containment strategies is required (Devlin *et al.,* 2006). According to Beaumont *et al.* (2009), only sterile GMO fish with no reproductive capability is thought to have a future in the aquaculture industry.

The probability of selectively bred farmed abalone escaping and settling in the wild is considered as low (Hawkins and Jones 2002; Theil *et al.,* 2004), but this has not been investigated as the industry is relatively young when compared to other animal production industries (Theil *et al.,* 2004). Due to the use of high volume continuous flow through production systems, the possibility of GM abalone escaping is a risk, and in South Africa there are no guidelines to manage and contain the potential escape of GMOs from commercial production systems.

The aim of this study was therefore to investigate the potential of biological, mechanical and chemical methods to contain GMO abalone (*Haliotis midae*) in commercial production systems, and the formulation of guidelines to mitigate the potential impact of GM abalone on the genetic diversity of natural populations along the South African coastline.

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# Chapter 2

# Literature Review

## 2.1 Commercial culture of abalone in South Africa

The high demand for abalone products from the East (Tarr, 1989; Britz *et al.,* 2009) led to the establishment of the South African abalone export industry in 1949. Commercial abalone farming started in 1990, and has since developed into the most valuable sector within the South African aquaculture industry. Restrictions on the harvesting of wild stocks led to a further increase in commercially reared abalone (Troell *et al.,* 2006). In 2012, 12 commercial abalone farms had been established along the coastline of South Africa (DAFF, 2012; Figure 2.1).

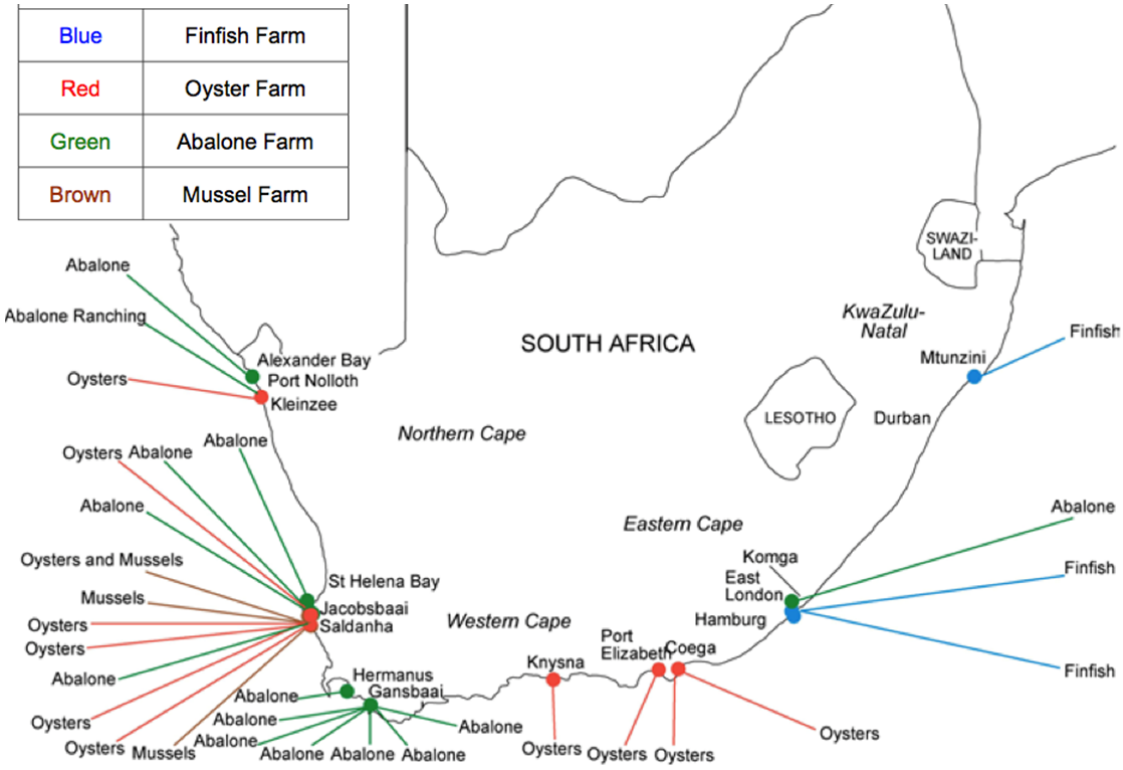



**Figure 2.1**. Location of abalone farms (Green) around the coast of South Africa (DAFF, 2012).

The national abalone industry contributes to the growth and stability of local and regional economies by the generation of income through export earnings, and the development of the socio-economic capacity of poor coastal communities in South Africa (Troell *et al.,* 2006). According to the Department of Forestry and Fisheries (DAFF, 2012), the abalone sector was valued at an estimated R357 million in 2011, which represented 94% of the entire country's aquaculture sector (DAFF, 2012).

The growth of the abalone industry in South Africa is due to collaboration between Government-backed research institutions and private sector stake holders (Sales and Britz, 2001). Factors that affect the viability and production efficiency of the abalone industry include spawning and seed production, growth, temperature, handling and transport, yield and quality, survival, nutrition, and the health of the animals (Prins, 2011). Feed makes up 60 - 70% of production costs of an abalone farm. A growth period of up to five years is required for the animals to reach the marketable size of 70 – 100 grams (Sales and Britz, 2001). Genetic modification through selection, and the use of biotechnologies such as triploid induction to increase growth rate may potentially assist in reducing the cost of production, and enhancing the profitability of the industry (Rasmussen and Morrissey, 2007).

## 2.2 Classification of *Haliotis midae*

The taxonomic classification of *Haliotis midae* is indicated in Table 2.1. Abalone belong to the Class Gastropoda, along with chitons, snails, octopuses, and oysters (De Beer, 2004; Heasman and Savva, 2007).

**Table 2.1.** Taxonomic classification of the abalone (*Haliotis midae*) (Hahn, 1989).

| | |
|---|---|
| Phylum | *Mollusca* |
| Class | *Gastropoda* |
| Subclass | *Prosobranchia* |
| Order | *Archaeogastropoda* |
| Family | *Haliotidae* |
| Genus | *Haliotis* |
| Species | *midae* |

## 2.3 Anatomy of *Haliotis midae*

All haliotids, including *Haliotis midae* (HM) are considered univalve herbivorous gastropods (De Beer, 2004). Haliotid anatomy typically includes a single flat shell, which is characterized by an enlarged body, whorl, and a reduced spire. Figure 2.2 gives a diagrammatic presentation of ventral view of abalone.

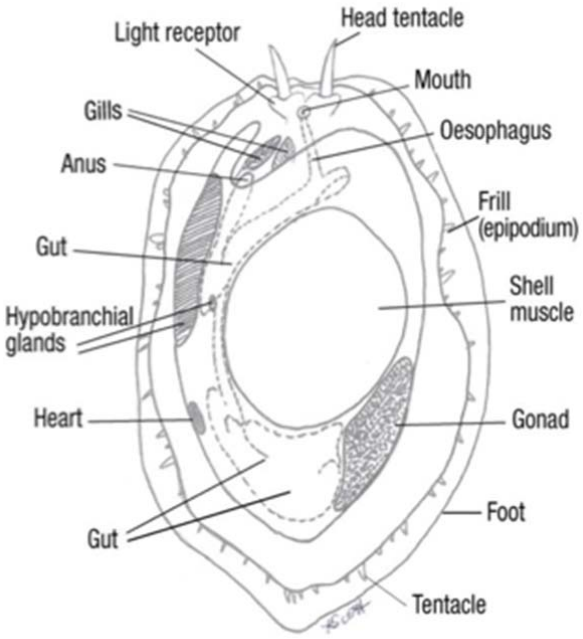



**Figure 2.2.** A diagrammatic presentation of the ventral anatomy of abalone (Visser-Roux, 2011).

A line of respiratory holes that perforate the ear-shaped shell is used for breathing and reproduction (Muller, 1986; Genade *et al.,* 1988; Hahn, 1989; Haesman and Savva, 2007). The soft body of the animal, which consists of the head, muscular foot, and visceral mass is located underneath the shell and is attached to the shell via the abductor or shell muscle. The various organ systems are positioned around the abductor muscle beneath the shell. The edge of the muscular foot is known as the epipodium, a sensory structure bearing tentacles. The foot of the abalone, which is the edible part, can contribute up to a third of the weight of the animal (Fallu, 1991). The crescent-shaped gonad surrounds the digestive gland, and collectively the two organs are known as the conical appendage (Figure 2.3).

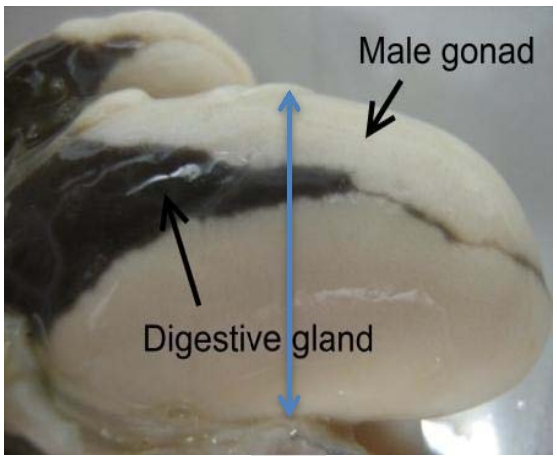



**Figure 2.3.** The conical appendage of a male abalone (*Haliotis midae* ), that consists of the gonad and the digestive gland (Visser-Roux, 2011).

Abalone species are dioecious, meaning each gender exhibits separate single gonads (Haesman and Savva, 2007; Visser-Roux, 2011). The gonads are characteristic in colour, with males typically displaying a creamy to white testis, and the female gonad being blue/green in colour (Figure 2.4). The gonad comprises about 20% of the animal's body weight during a breeding season, after which a rapid decrease in size can be observed post spawning (Hahn, 1989; Henry, 1995). The latter condition is referred to as "spent".


**Figure 2.4.** The characteristic gonad colour of *Haliotis midae*, A: indicating the blue-green colour of the ovary, and B: showing the creamy-white colour of the testis (Visser-Roux, 2011).

According to Visser-Roux (2011), it is difficult to distinguished between male and female HM individuals before the age of one year. A definite gonadal cavity can be seen in HM at one year of age, but no sex cells can be identified at this stage of development. At one and a half years of age, gender differentiation is possible using histology, as the gonad cavity is filled with gametogenic cells (Visser-Roux, 2011). At three years of age (i.e. 70 – 120 mm in shell length), gonads are macroscopically distinguishable, with the colour of the gonads making it relatively easy to determine the gender of the animal (Figure 2.4).

## 2.4 Reproduction in *Haliotis midae*

### 2.4.1 Seasonal nature of reproduction in *Haliotis midae*

Reproductive cycles vary between abalone species, and are largely influenced by water temperature (Newman, 1967; Litaay and De Silva, 2003). Wild HM spawn twice a year during spring (September-October), and autumn in the Southern Hemisphere (April-May) (Brits and Sales, 2001; Lucas *et al.,* 2005). Spawning is triggered by a number of external factors, including air exposure, the lunar cycle, photoperiod, and the upwelling of nutrients (Purchon, 1977; Hahn, 1989; Fallu, 1991).

### 2.4.2 Puberty and sexual maturity in *Haliotis midae*

The egg-laying behaviour of gastropods is regulated by an egg-laying hormone (ELH). Egg-laying hormone therefore plays an essential role in spawning induction (Ngernsoungnern *et al.,* 2009). A neuromodulator, known as APGWamide is associated with the ovulation and spermiation of abalone (Chansela *et al.,* 2009). Shell growth and certain metabolic pathways are regulated by a molluscan insulin-like peptide (MIP) (Taylor *et al.,* 2008), which indirectly affects the reproduction of abalone.

Abalone species differ in the chronological age at which they attain sexually maturity (Shepherd and Laws, 1974, Visser-Roux, 2011). Sexual maturity in abalone is characterized by males and females producing spermatozoa or primary oocytes respectively, which can be clearly distinguished in the gonadal tissue (Hahn, 1989). In wild-caught abalone, it is suggested that sexual maturity occurs at seven years of age (Newman 1967), although it is found to occur earlier in animals living in warmer waters, with the latter group attaining sexual maturity as early as three years of age (Wood and Buxton, 1996), such as abalone found along the east coast of South Africa.

When male and female abalone have attained sexual maturity, they will be able to spawn on cue of appropriate stimuli. Spawning is the release of large numbers of gametes into the ocean, with no protection for the gametes or the developing animals (Purchon, 1977). Spawning is initiated predominantly by an increase in water temperature, and females are also induced to spawn by the presence of male gametes in the water. The oocytes expelled from the gonad are stage of Meiosis I. Meiosis II is therefore considered a developmental stage, as it only occurs after fertilization (Hahn, 1989; Guo *et al.,* 1992; De Beer, 2004).

### 2.4.3 Oogenesis in *Haliotis midae*

In sexually mature female HM, a seasonally influenced reproductive cycle can be divided into four distinct stages, i.e. gametogenesis, vitellogenesis, oogenesis, and spawning. Environmental cues play an important role in the initiation of reproductive activities in abalone, with a change in water temperature being able to initiate gametogenesis, and an increase in food supply initiating oocyte growth and vitellogenesis (Hahn, 1989).

According to Newman (1967), Hahn (1989) and Visser-Roux (2011) the HM ovary is compartmentalized and separated by the trabeculae (Figure 2.5). The trabeculae consists of sheets of connective tissue that support the germinal epithelium, the site of oogenesis development. The lumen of a fully developed (sexually mature) gonad contains mature ova embedded in a gelatinous matrix.

Oogenesis in female HM is classified into nine distinct stages as shown in Table 2.2 (Visser-Roux, 2011).

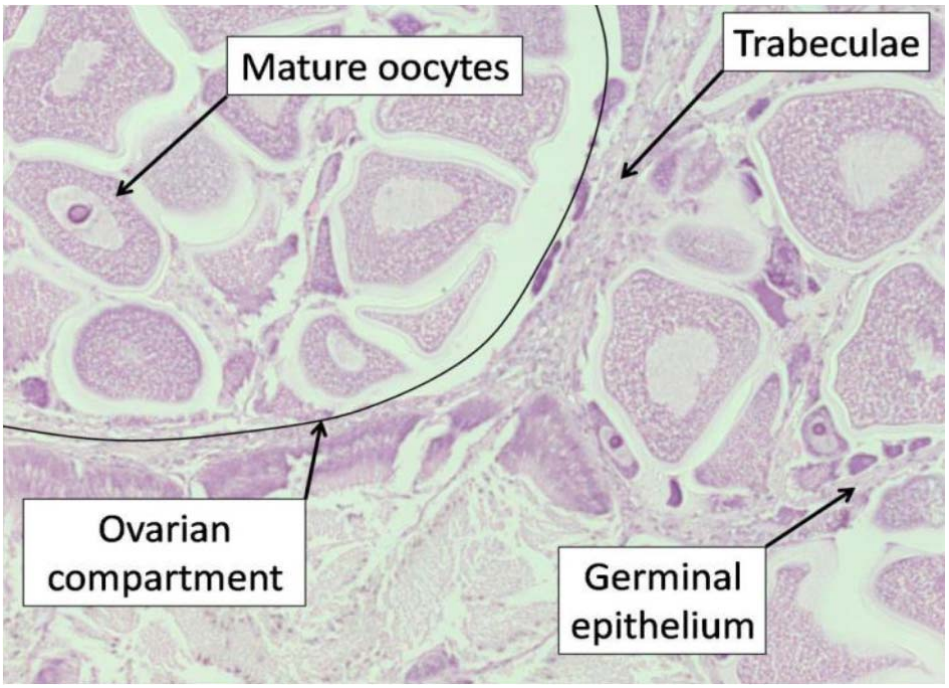



**Figure 2.5.** A micrograph of a *Haliotis midae* ovary, with trabeculae, the germinal epithelium, and mature oocytes visible in an ovarian compartment (10X magnification; Visser-Roux, 2011).

Primordial oogonia are found attached to the trabeculae, and as they gradually undergo maturation, they move progressively away from the trabeculae, and into the ovarian compartment. Female gametes increase in size as they mature, in contrast to male gametes that decrease in size (Visser-Roux, 2011).

**Table 2.2.** A description of the nine stages of oogenesis that can be observed in *Haliotis midae* gonads (Visser-Roux, 2011).

| Stage | Description |
|---|---|
| Oogonia | Oogonia are the first developmental stage of mature oocytes, and normally occur attached to the trabeculae (Og; Figure 2.6A). Squamous-shaped follicular cells surround the oogonia and on average have a diameter of 15 to 20 µm. The nucleus of an oocyte is approximately 10 µm in size, and stain light-blue haematoxylin-eosin. The dark blue cytoplasm of oocytes indicates the presence of ribosomes. |
| Stage 1 oocytes | Oogonia develop into Stage 1 oocytes, which are more oval-shaped and still attached to the trabeculae (St1; Figure 2.6B). Stage 1 oocytes range between 20 to 25 µm in diameter, with the nucleus appearing light blue, showing the densely packed chromatin within the nucleus when stained with haematoxylin-eosin. |
| Stage 2 oocytes | The presence of lipid droplets differentiates Stage 2 oocytes (St2) from Stage 1 oocytes (Figure 2.6C). Stage 2 oocytes are characteristically 30 to 35 µm in diameter, with a lightly stained nucleus, when stained with haematoxylin-eosin. The average size of the nucleus of Stage 2 oocytes is 20 µm. |
| Stage 3 oocytes | Stage 3 oocytes increase to an average size of 45 to 55 µm, and assume scallop shape (St3; Figure 2.6D). The nucleus appears clear due to uncondensed chromatin. The chorion starts to develop around the egg; a higher density of lipid droplets is seen in the cytoplasm. |
| Stage 4 oocytes | Stage 4 oocytes are approximately 60 to 80 µm in diameter, with the nucleolus still staining dark. Lipid droplets within the cytoplasm become more prominent (St4; Figure 2.6E). At this stage of development, the oocytes are still attached at the base to the trabeculae, and the chorion becomes more visible. |

| | |
|---|---|
| Stage 5 oocytes | Stage 5 oocytes assume a more columnar shape, ranging from 100 to 150 µm in size (St5; Figure 2.6F). The chorion becomes increasingly visible, and the cytoplasm is characterized by high density of lipid droplets, as well as the presence of yolk platelets. The nucleus appears almost completely transparent, with the nucleolus stain lighter. |
| Stage 6 oocytes | Stage 6 oocytes are elongated, and are attached by means of the cytoplasmic stalk to the trabeculae (St6; Figure 2.6G). The chorion becomes encased within a thick jelly-like coat, which separates cells into loose cell clusters. |
| Stage 7 oocytes | Stage 7 oocytes are now oval in shape, with the cytoplasmic stalk being completely separated from the trabeculae (St7; Figure 2.6H). Stage 7 oocytes lie freely within the lumen, with the chorion and a thick jelly-coat surrounding each cell. |
| Stage 8 oocytes | Stage 8 oocytes are considered to be mature, and ready to be spawned. Mature oocyte size varies between 260 and 300 µm, and the nucleus appears completely clear with nuclear chromatin that is unravelled. Lipid droplets are found in large clusters making the cytoplasm appear clear (St8; Figure 2.6I). |

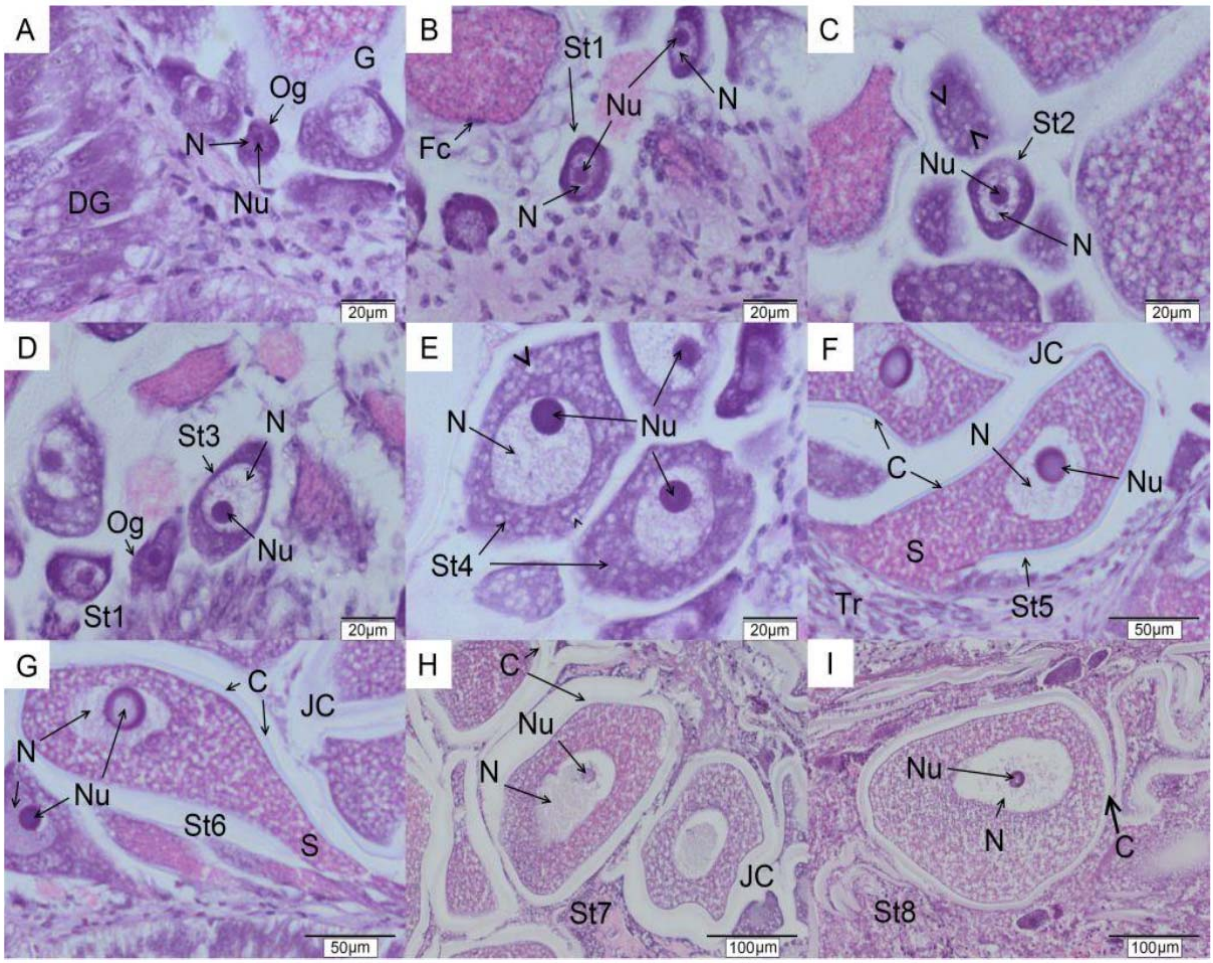



**Figure 2.6.** Oogenesis in *Haliotis midae* (C = chorion; DG = digestive gland; FC = follicular cell; GEp = gonad epithelium; JC = jelly coat; N = nucleus; Nu = nucleolus; Og = oogonia; S = cytoplasmic stalk; St1 = stage 1 oocyte; St2 = stage 2 oocyte; St3 = stage 3 oocyte; St4 = stage 4 oocyte; St5 = stage 5 oocyte; St6 = stage 6 oocyte; St7 = stage 7 oocyte; St8 = stage 8 oocyte; > = lipid droplets) (Visser-Roux, 2011).

### 2.4.4 Spermatogenesis in *Haliotis midae*

The male HM gonad is characterized by densely packed tubules of connective tissue, lined with germinal epithelium, the site of spermatogenesis (Figure 2.7). The germinal epithelium is supported by the trabeculae. As spermatogenesis progresses, the spermatogonia undergo transformation, and move further away from the trabeculae, and into the lumen. When HM males are sexually mature and able to spawn, millions of spermatozoa can be observed in the lumen of the tubules (Figure 2.7).

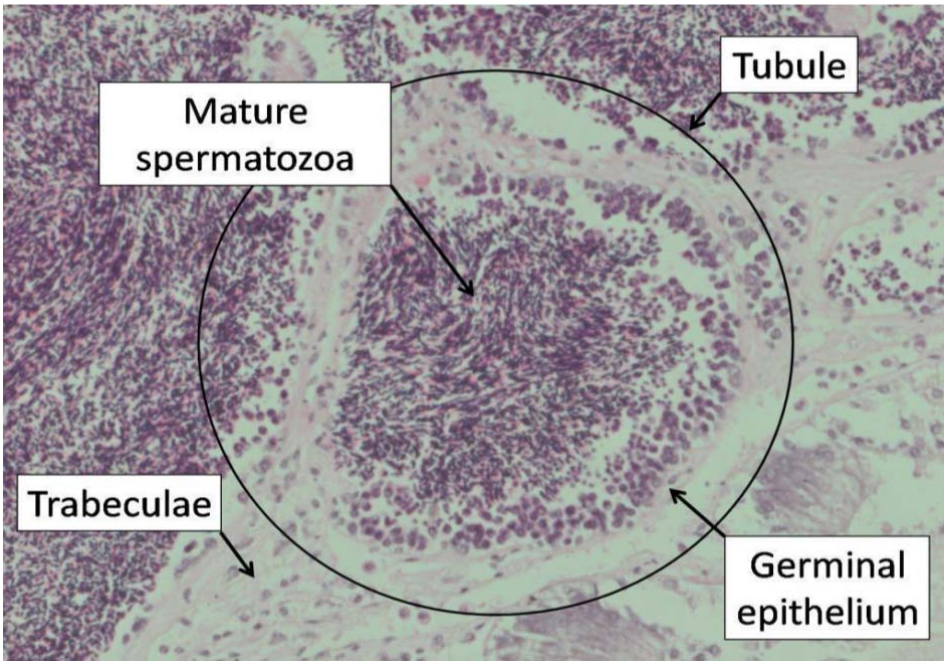


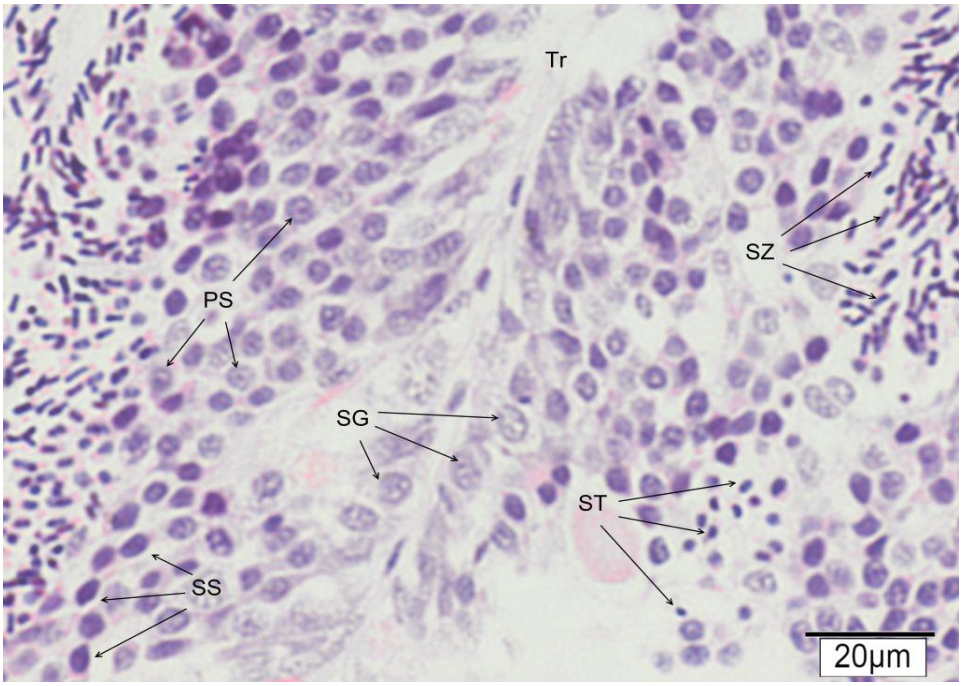

**Figure 2.7.** A micrograph of a *Haliotis midae* gonad, indicating mature spermatozoa in a tubule lined with germinal epithelium (magnification X10; Visser-Roux, 2011).

Spermatogenesis was initially categorized into four stages by Newman (1967) and Wood and Buxton (1996), but a more recent study by Visser-Roux (2011), indicated that there are five distinct stages of spermatogenesis observable in HM (Table 2.3). Newman (1967) and Wood and Buxton (1996) based their description on cell size, whereas Visser-Roux (2011) used histological characteristics to classify the respective stages of the spermatogenesis process.

**Table 2.3.** A description of the five stages of spermatogenesis that can be observed in *Haliotis midae* gonads (Visser-Roux, 2011).

| Stage | Description |
|---|---|
| Spermatogonia | The first developmental stage, which are closely attached to the trabeculae (SG; Figure 2.8). Spermatogonia are oval-shaped, with an average diameter of 5 to 7 µm. The Spermatogonium nucleus exhibits a clear nucleus with some blue staining which indicates the uncoiled euchromatin. |
| Primary spermatocytes | Occur further away from the germinal epithelium, towards the lumen. Primary spermatocytes (PS; Figure 2.8) are characterized by increasing amounts of dark stained, dense heterochromatin within the nucleus that has an average size of 2.5 µm. Primary spermatocytes have an average diameter of 3 to 5 µm (Visser-Roux, |

| | |
|---|---|
| | 2011). |
| Secondary spermatocytes | Appear loosely attached to the trabeculae (SS; Figure 2.8), and are on average 5µm in length. Secondary spermatocytes stain darker, with a darker stained nucleus than the primary spermatocyte. These cells assume a more oval shape than the primary spermatocytes. |
| Spermatids | Occur in the lumen, and are completely detached form the trabeculae (Tr; Figure 2.8). Spermatids appear oval (ST; Figure 2.8), and have an average diameter of 2.5 to 3.5 µm. The nucleus show complete condensation of the heterochromatin, appearing very dark after staining |
| Spermatozoa | The final stage of spermatogenesis and have an average length of 2.5 to 3.5 µm. Spermatozoa (SZ; Figure 2.8), occur within the lumen of the gonad, and are considered ready to be released during spawning. |




.**Figure 2.8.** A micrograph of a *Haliotis midae* gonad indicating the five stages of spermatogenesis. (Tr = trabeculae, SG = spermatogonia, PS = primary spermatocytes, SS = secondary spermatocytes, ST = spermatids, and SZ = spermatozoa (magnification X40; Visser-Roux 2011).

### 2.4.5 Artificial spawning and fertilization in *Haliotis midae*

Wild HM only spawn twice a year, therefore the artificial manipulation of spawning induction is required for the commercial production of abalone.

Sodium hydroxide is used as the initial step to increase the pH of the water; hydrogen peroxide is then added 15 minutes later (Visser-Roux, 2011). Three hours later, tanks are cleaned of any residual hydrogen peroxide, which is harmful to the gametes (Morse *et al.,* 1977). Tanks are then re-filled with clean filtered seawater, and a constant airflow is maintained.

Males will begin spawning approximately four hours post induction. A volume of sperm from random male tanks is then added to the female tanks, which begin spawning two hours later (six-hours post induction).

The addition of sperm is said to increase spawning activity (Paulet, 2011). Sperm and ova are left to fertilize within the broodstock tanks.

## 2.5 Layout, management and commercial protocols of an abalone culture system

Abalone can be grown in land-based systems or in suspended cages at sea. In South Africa, land-based systems are used to rear HM. A typical HM culture system comprises of a hatchery and a grow-out section, with the former that includes a broodstock housing section, a larvae housing section, a settlement housing section, and a weaning section, which houses spat (See Figure 2.9). Due to the Farm's (HIK) proprietary information, stocking densities, certain time periods, flow rates of housing sections and other sensitive information cannot be disclosed.

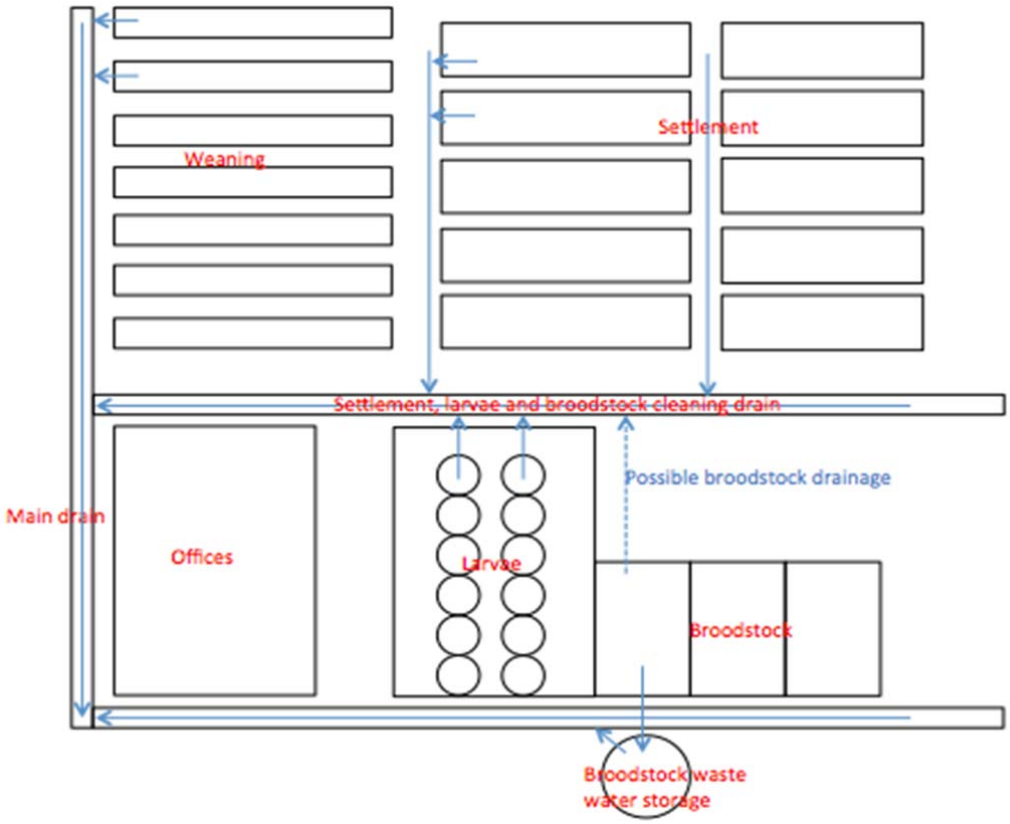



**Figure 2.9.** A diagram depicting the different sections of the HIK hatchery as well as the flow pattern of water as it exits each housing section to flow to the ocean (Paulet, 2011).

Male and female HM broodstock are kept in isolated rooms at a constant holding water temperature. Broodstock are induced to spawn to produce new larvae on a fortnightly basis. Animals are spawned, fertilised, settled, and weaned. Once spat are of the correct size, they are transferred to the grow-out section of the farm. The grow-out section is where animals are housed for three to five years, allowing them to grow to a harvestable size of 70 - 100 grams, at which size the animals are considered ready for export.

The farms water supply is typically a flow-through system, with the flow rate determined by the life stage housed in each specific section of the system. Water on the farm is pumped from the ocean, and into a header tank. The header tank filters all sand particles, after which the sand-free water is filtered through a 90 micron sieve. The filtered water is distributed to all sections of the farm. At each section, depending on the filtration requirement, the water will be filtered further before distribution to the tanks in that specific section.

Each of the separately housed life stage sections has inflow and outlet pipes that drain into localised cement drains within each section. It was for this reason that it was chosen to contain each of the life stages separately in an already existing system on the farm, as the total volume of water is too large to try and contain at a final point of exit on the farm.

Farms are equipped with bio-secure areas, which are implemented between all the housing stages to prevent the spread of disease and cross contamination of sections, should it occur in a specific section (Paulet, 2011). Bio-secure areas include a hand washing basin and a footbath containing sodium hypochlorite.

### 2.5.1 Broodstock housing section

The broodstock housing section is the most critical point for escapees, with the simultaneous spawning of HM males and females, and the direct access to the ocean. Plastic tanks house the broodstock, and are arranged on two levels on either side of the broodstock room. Tanks are supplied with aerated filtered seawater, and kept in isolated rooms at a constant holding temperature. Broodstock are kept at densities ranging from one, three, or five animals per tank.

Wastewater drains from each tank into a plastic central gutter that runs along the front of the tanks on either side of the broodstock room. The effluent water then flows into cement drains, which join onto the farm's main drain. From here water flows back to the sea. Currently there are no methods to contain, and thus to prevent HM male and female gametes from escaping into the ocean with the effluent water. Ova range between 260 - 350 µm in size, and sperm are on average 2.5 - 3.5 µm in length, and 0.5 µm in diameter (Visser-Roux, 2011).

### 2.5.2 Larvae housing section

The larvae section is the second critical point for potential escapees to occur. Larvae have not yet settled, and are able to swim with the outflow of water into the ocean, potentially settling in the vicinity of natural stocks.

The larvae are maintained in cone-shaped fiberglass tanks housed in a separate room. Tanks are supplied with a constant flow of air, and filtered seawater. The fiberglass tanks are open at the top, with the cone facing downwards over a cement drain. This cement drain links to the main farm drain, allowing wastewater

to flow directly back to sea. Currently each larvae tank is fitted with a banjoe sieves (i.e. an overflow pipe covered with 120 µm mesh), which serves as a method of containment.

Larvae are maintained in this section until they metamorphose from veliger larvae to trochophore larvae (Figure 10), which takes approximately five days (Paulet, 2011). Larvae are lecithotropic, surviving on their yolk sacs. Once the yolk sacs have been absorbed, larvae begin swimming up in search of food, at which point larvae are ready to settle. Veliger larvae (Figure 2.10) range between 300 - 350 µm in size, and trochophore larvae (Figure 2.10) are on average 300 µm in size (BHCAP, OceanLink, 2012).

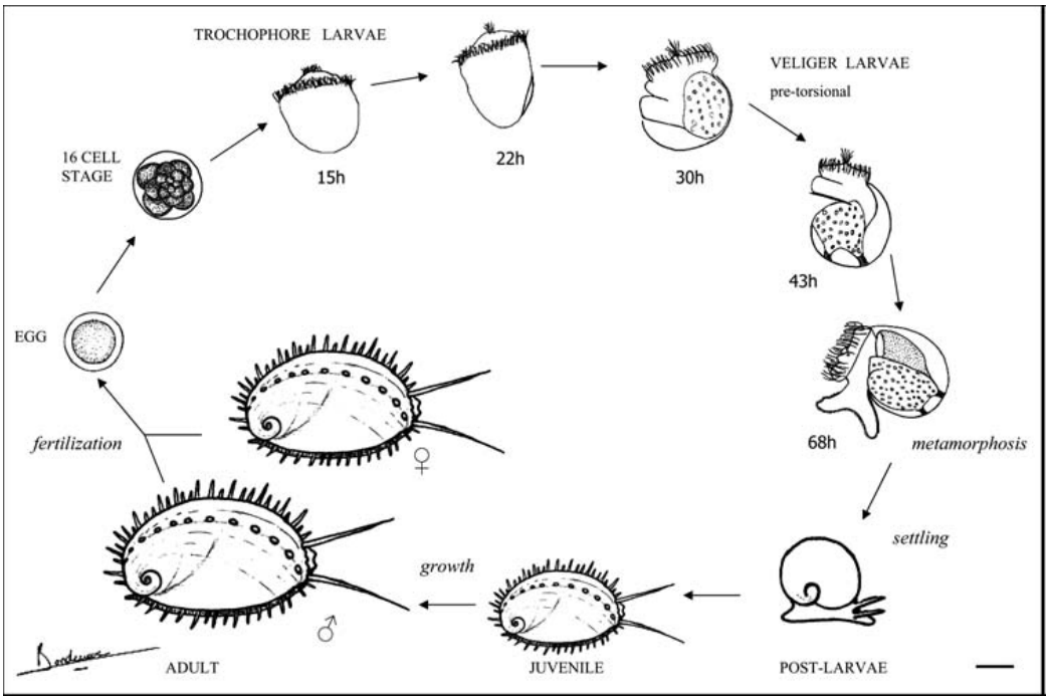



**Figure 2.10** The different life stages of the abalone *Haliotis tuberculata*, from larval to adult stages (Jordiller *et al.* 2008).

### 2.5.3 Settlement housing section

The settlement section of the farm is not considered a critical point of escapees. The settlement section contains rectangular plastic tanks, with sheets of plastic corrugated sheets coated in diatoms placed vertically in the tanks at specific intervals (Figure 2.11). Diatoms serve as a nutrition source for the young abalone as shown in Figure 2.11. The settlement tanks are changed to closed systems for a short duration after the addition of larvae to the tanks. After the closed period, the water is then allowed to flow at a specific rate through the tanks. Larvae that have not settled on the diatom plates have an unknown chance of surviving and settling in the wild. Hawkins and Jones (2002) and Theil *et al.* (2004) hypothesized that the probability of escaped larvae settling in the wild is low. This has not been investigated as this industry is considered to be relatively young when compared to other animal production systems (Theil *et al.,* 2004), and thus must still be investigated.

Each settlement tank is fitted with a standpipe positioned above a cement drain. Effluent water flows over, and out the standpipe into the cement drains, linking to the main farm drain and flowing back into the ocean. Currently there are no means of containing the settlement animals, presenting a potential additional critical point.

**Figure 2.11.** A typical settlement tank in an abalone hatchery (DAFF, 2011).

Larvae undergo a metamorphic phase between five to seven days (Figure 10), depending on the sea water temperature. Once metamorphosed, animals will attach themselves to the diatom covered plates, and start to feed. At this stage settled larvae are referred to as spat. Settlement larvae range between 350 - 700 µm in diameter (BHCAP OceanLink, 2012).

### 2.5.4 Weaning section

The weaning section of a hatchery, which houses spat, is the third critical point of escape. Spat have a tendency to crawl out of the tanks at night, and make their way into the drains that link to the ocean.

The weaning section consists of rectangular plastic tubs, equipped with cones (Figure 2.12), and supplied with a constant flow of air, and water at ambient temperatures. Weaning tanks are elevated on wooden structures, 1 m above the cement drains. Each tank is fitted with a standpipe, positioned above the cement drain. Effluent water flows out the tanks, into the cement drain, which in turn links to the main farm drain, with the latter that transports the water back to the ocean.

**Figure 2.12.** A typical weaning tank in an abalone hatchery (DAFF, 2011).

Currently a 0.3 cm mesh is placed over the standpipe of the weaning tank to prevent animals from crawling down the pipes, but no means to contain the animals from escaping over the sides of the tank is used. Spat remain in settlement tanks for a period of time, and are then transferred to the weaning area. Spat range in size from 700 - 1000 µm (BHCAP OceanLink, 2012).

## 2.6 Application and impacts of genetics modification technologies

### 2.6.1 Genetic modification and animal production

Genetically modified organisms (GMOs) are organisms with an altered genetic make-up to improve specific production traits (Morse, 1986; Wong and Van Eenennaam, 2008), e.g. an enhanced resistance to herbicides, or an improvement in nutritional content (Whitman, 2000). The use of artificial selection, and cross-breeding have been used in the agricultural industry for many years (Phillips, 2008), but research into genetic engineering has allowed for the introduction of accurate control over the genotypes of organisms for improved commercial products (Phillips, 2008). Genetically modified organisms are found in a wide variety of industries such as viticulture (e.g. genetically improved yeast (Dequin, 2001), agronomy (e.g. crop disease resistance and seedless fruit), and the manufacturing of pharmaceuticals e.g. (vaccines) (Piferrer *et al.,* 2009).

In the livestock industry, biotechnologies such as artificial insemination and gene transfection, have been used to improve the production and reproduction efficiency of animals. In recent years, an increased emphasis on livestock producers to farm cost-effectively, and have also highlighted that production practices need to be sustainable with a minimized impact on the surrounding environment (Hallerman and Kapuscinski, 1995; Beardmore and Porter, 2003).

### 2.6.2 Transgenics in the aquaculture industry

The production of aquatic GMOs that show significant increase in growth rate, presents an opportunity to promote the cost-efficient, and effective use of aquaculture species to indirectly address food security, and poverty alleviation issues (Hallerman and Kapuscinski, 1995; Beardmore and Porter, 2003).

The use of micro-injecting an exogenous gene into fertilized ova has been the most widely used method of producing transgenic fish (Maclean and Laight, 2000). Transgenic fish species have shown properties that are beneficial to the growth of the aquaculture industry, and include increased feed conversion efficiency, disease resistance, cold tolerance, increased carcass yields, increased protein levels, and reduced fat content (Maclean and Laight, 2000; Beardmore and Porter, 2003; Devlin *et al.,* 2006; Hu *et al.,* 2007; Wong and Van Eenennaam, 2008; Dunham, 2009). The biotechnological techniques used to produce transgenic fish for aquaculture also include the design and construction of transgenic DNA, transfer of the gene construct into the fish germ cells, study of inheritance, and the selection of stable lines of transgenics (Levy *et al.,* 2000).

The use of GMOs to improve the overall production efficiency of commercial systems will ultimately be determined by how effectively these GMOs can be contained (Wong and Van Eenennaam, 2008). A potential approach to ensure effective containment is to create sterile organisms, as these organisms would not be able to contribute to the genetic diversity of an ecosystem (Allen *et al.,* 1986; Cal *et al.,* 2006). Salmon farms have considered using a sterile fish (triploid) as a means to contain salmon from escaping the commercial production units, as farmed salmon have negative impacts on the genetic diversity and the ecology of the natural environment (Cotter *et al.,* 2000). The triploid Atlantic salmon, (*Salmo salar*) is considered a functionally sterile organism, and producing it in an intensive farming system would potentially reduce the threatening interactions between wild and farmed fish, and reduce the ecological impact on the environment (Oppedal *et al.*, 2003).

By 2006 more than 30 fish species had been genetically engineered, including many prominent aquaculture species such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), zebrafish (*Danio rerio*), and coho salmon (*Oncorhynchus kisutch*). Table 2.4 shows some of the target phenotypes in key aquaculture species using a specified transgene.

**Table 2.4.** The application of genetic modification (i.e. transgenes) to obtain desired phenotypic effects in aquatic species (Devlin *et al.,* 2006).

| TARGET PHENOTYPE | TRANSGENE | SPECIES |
|---|---|---|
| Growth | Growth hormone | Atlantic salmon, *Salmo salar*<br>Rainbow trout, *Oncorhynchus mykiss*<br>Coho salmon, *Oncorhynchus kisutch*<br>Chinook salmon, *Oncorhynchus tshawytscha*<br>Rohu, *Labeo rohita* |
| Freeze Tolerance | Anti freeze protein | Atlantic salmon, *Salmo salar* |
| Disease resistance | Lecropin<br>Lactoferrin<br>Lecropin | Catfish, order *Siluriformes*<br>Carp, *Cyprinus carpio*<br>Medaka, *Oryzias latipes* |
| Carbohydrate metabolism | Glucose transporter<br>Hexokinase | Rainbow trout, *Oncorhynchus mykiss* |
| Reproduction | Antisense GnRH | Rainbow trout, *Oncorhynchus mykiss* |
| Lipid metabolism | D6-desaturase | Zebrafish, *Danio rerio* |
| Phosphorus metabolism | Phytase | Zebrafish, *Danio rerio* |
| Vitamin C Metabolism | L-gulono-gamma-lactone oxidase | Rainbow trout, *Oncorhynchus mykiss* |

Antifreeze protein (AFP) and growth hormone (GH) have been used as transgenes, and are classified as two exogenous proteins that would benefit the aquaculture industry (Maclean and Laight, 2000). Antifreeze protein is believed to improve the cold resistance of a species (previously experimented and successful in goldfish, (*Carassius aurafus auratus*)), which would allow for the production of the species outside of their natural climatic zone (Beardmore and Porter, 2003; Rasmussen and Morrissey, 2007). The use of growth hormone in salmonids exhibited growth rates of up to three to five times the size of non-transgenic salmonids (Cook *et al.,* 2000; Melamed *et al.,* 2002; Devlin *et al.,* 2006). The production of faster growing fish in geographical locations previously not acceptable for aquaculture would potentially initiate a bigger growth in

the industry (Le Curieux-Belfond *et al.,* 2009). Aquatic species have definite temperature requirements, this could aid in the diversification of species farmed around the world.

The application of transgenesis is, however, associated with negative effects. Species treated with the transgenes (AFP and GH) have shown both positive and negative pleiotropic effects related to the morphology (e.g. acromegaly), physiology, metabolism, immunology, and behaviour (e.g. overt aggression) of transgenic organisms (Devlin *et al.,* 2006; Le Curieux-Belfond *et al.,* 2009). Transgenic salmonids have exhibited an altered skeletal structure, immune function (Kim *et al.,* 2013), impaired swimming ability (Farrell *et al.,* 1997), a reduced disease resistance (Kim *et al.,* 2013), altered oxygen utilization and metabolism, altered gonadal development, altered anatomy of the gut, gill, muscle, heart and liver structures, and altered regulation of growth and insulin-like growth factor I (IGF-1) hormones (Devlin *et al.,* 2006). Major behavioural changes can also occur in transgenic animals, e.g. enhanced feeding, reduced discrimination of prey choice, reduced schooling tendency, and increased predation mortality, which may all affect the ability of the salmon to reproduce and survive in nature (Devlin *et al.,* 2006).

The acceptance and incorporation of the abovementioned transgenes is variable. It is not guaranteed that the genes in question will be expressed in the environment, as the control of the expression of the transgene is unpredictable (Maclean and Laight, 2000; Le Curieux-Belfond *et al.,* 2009). Further research is necessary to establish the expression of genes in transgenic organisms, as GMOs that express the target gene will be of interest to the aquaculture industry (Beardmore and Porter, 2003; Dunham, 2009; Le Curieux-Belfond *et al.,* 2009). The same research applies to the inheritance of transgenic genes that are expressed at an unacceptable level, and the ability of these transgenic animals to transmit the gene to their progeny (Beardmore and Porter, 2003).

Crevel *et al.* (2002) stated that the ingestion of AFP via a fish protein source has no adverse effect on humans, but as with any foodstuff, the potential of the development of allergies should not be ignored, and further investigations are necessary.

#### *2.6.2.1 Further uses of transgenic aquatic organisms*

Various other uses that would positively benefit the aquaculture industry have been proposed for GMOs (Beardmore and Porter, 2003). By incorporating the use of pollution sensitive promoters in transgenic animals, such species can monitor the pollution within the animal's environment. This may include the use of a green fluorescent protein structural gene (GFP), which will be inactivated by the presence of heavy metals, resulting in a visible colour change (Le Curieux-Belfond *et al.,* 2009). Another example is the use of AFP to preserve sheep and pig embryos by protecting the membranes from freezing. This could be of use in the aquaculture industry in preserving transgenic lines, and supplying new hatcheries and farms with suitable stocks (Beardmore and Porter, 2003). In the ornamental fish industry, the genes encoding for fluorescent proteins in a wide variety of colours have become available, thus allowing for novel coloured fish being produced for the ornamental fish market (Melamed *et al.,* 2002).

*2.6.2.2 Commercial impact of genetically modified organisms*

The proposed improved growth rate of commercially produced triploid abalone and all other advantages associated with transgenic species will potentially contribute to increasing the income, and improving the production efficiency, of the aquaculture industry. It is important that aquaculture production systems are managed optimally to ensure the cost-efficient production of marketable animals (Duame, 2003).

An increase in growth rate of triploid abalone will have a direct impact on production protocols, with a shortened grow-out to slaughter interval (Piferrer *et al.,* 2009), which in turn will affect the holding time as well as decrease the feed requirements of abalone from hatch to slaughter. Figure 2.13 indicates the functional genomic and transgenic technologies, and their potential benefit to the aquaculture industry (Melamed *et al.,* 2002).

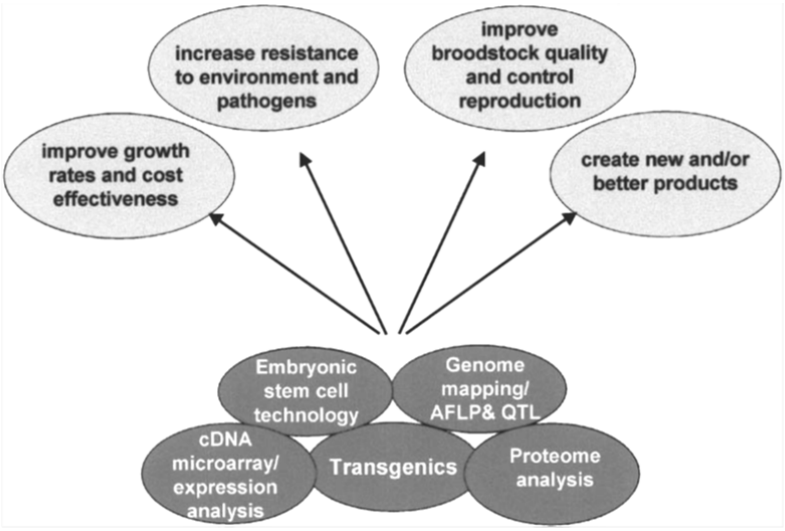



**Figure 2.13.** The various functional genomic and transgenic technologies, and their potential benefit to the aquaculture industry (Melamed *et al.,* 2002).

The advantages associated with transgenic organisms which will impact the aquaculture industry include an increased disease resistance to pathogens, which could minimize the loss of animals often experienced in farming practices, thereby directly benefiting the welfare of the animals and contributing towards higher profits. The settlement rate on abalone farms range between 30 - 60% (Duame, 2003), and a potential improvement in settlement rate by means of transgenic biotechnologies will directly impact on the output yield of abalone farms. Sterile triploid abalone would also not experience the typical side effects on meat quality when stressful spawning occurs during shipping (Brake *et al.,* 2004). Transgenic animals, when marketed correctly, would allow a quality product all year round, even during the spawning season (Brake *et al.,* 2004). The creation of new and/or better products, for example, animals with higher carcass yield or protein levels combined with decreased fat, will result in higher profits for the industry.

*2.6.2.3 Environmental impact of genetically modified organisms*

Risk is defined as the possibility of harm (e.g. a negative effect on a prey population) resulting from an exposure to a hazard (i.e. increased feeding; Maclean and Laight, 2000; Devlin *et al.,* 2006). In aquaculture systems, exposure of animals to hazards can be considered as a two-step process, firstly the escape of an animal from a culture facility, and secondly the resulting introduction of the transgenic gene into the wild population (Devlin *et al.,* 2006). If a transgene is unable to sustain itself within a given population, the effect of the transgene is considered short-term and manageable (Devlin *et al.,* 2006). Sterile triploid abalone would be considered a manageable risk, as they would not attain sexual maturity and interbreed with the wild population.

Poor management associated with the risk of releasing genetically enhanced animals into the wild is difficult to predict. Genetically modified animals are considered a hazard if a transgene was integrated into a wild population (Muir and Howard, 1999; Maclean and Laight, 2000; Hu *et al.*, 2007), which would result in the genetic integrity of the domestic wild stocks being compromised (Hawkins and Jones, 2002). The viability of progeny resulting from matings of GM farmed and wild organisms is unknown, and can potentially result in the extinction of both wild and transgenic populations (Muir and Howard, 1999).

Risk assessment involves the identification and analysis of who would be affected by the escape of GMOs into the natural environment (i.e. effect on societal groups, genetic diversity and ecological effects), as indicated in Figure 2.14 (Hallerman and Kapuscinski, 1995).

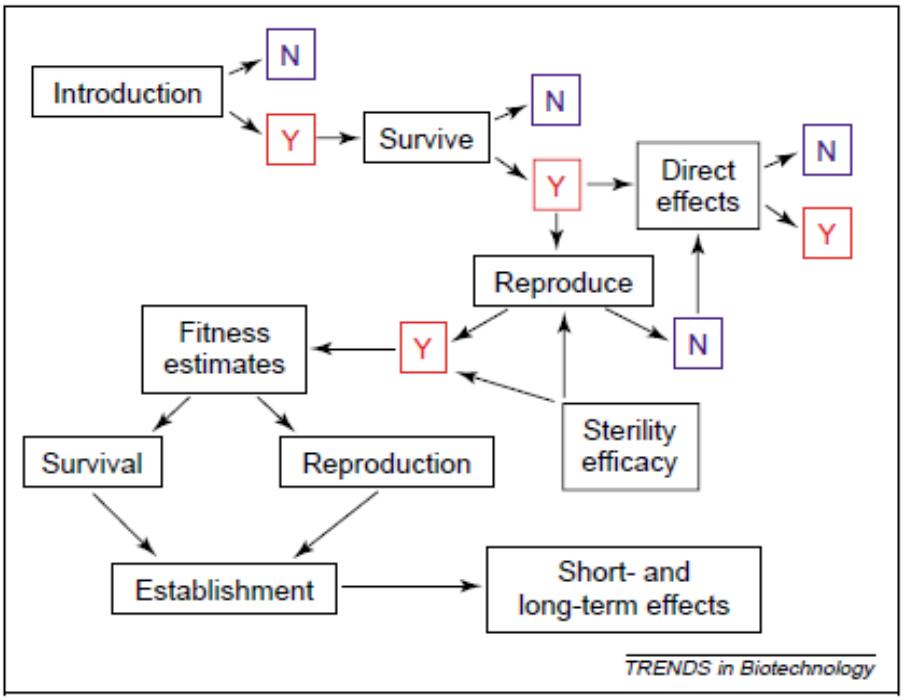



**Figure 2.14.** Diagram showing a simplified model outlining the risk assessment process involved in the introduction of trasngeic animals into nature (Devlin *et al.,* 2006).

An example of the ability of a transgenic organism to out-compete the wild population for their natural environment is the smooth cod grass (*Spartina maritime*) in England. The smooth cod grass was introduced from the east coast waters to Southampton, where it hybridized with endemic grasses to produce a sterile hybrid *Spartinia townsendii,* which underwent amphidiploidy and produced a tetraploid species *Spartinia anglica.* The hybridized species displayed fast growth, high fecundity and rapid ability to colonize. The grass then began to dominate mud flats which reduced the preferred available habitats of local birds, which in turn were now unable to feed and roost (Piferrer *et al.,* 2009). This example portrays the risk involved in the release, or even the relocation of a species, and the damage they are able to cause in a given ecosystem. Other examples of invasive species are presented in Table 2.5.

**Table 2.5.** A table showing examples of alien species out competing native species (Wildlife Conservation, 2012).

| Species | Description of Invasion |
|---|---|
| Water hyacinth | An aquatic plant that was introduced into the United States as an ornamental plant from South America. The plant forms dense mats, reducing sunlight for submerged plants and other aquatic organisms and causing clogging of waterways. |
| European green crabs | Out-competing native species of the San Francisco Bay for food and habitat. The crabs eat huge quantities of native shellfish, threatening commercial fisheries. |
| Zebra mussels | Originally from Europe, Zebra mussels attached themselves to ships and ended up in the Great Lakes. Zebra mussels and quagga mussels are virtually identical both physically and behaviourally and the United States is experiencing a devastating invasion of these mussels. |
| Asian carp | Asian carp, which were introduced into southern fish farms, have spread quickly across the United States. Asian carp are fast growing, aggressive and adaptable fish that are out competing native fish species for food and habitat in much of the mid-section of the United States. |

### 2.6.3 Ploidy

*2.6.3.1 Triploidy*

Triploids can spontaneously occur in the wild and cultured stocks (Cal *et al.,* 2006; Piferrer *et al.,* 2009). The occurrence of triploidy in the wild occurs predominantly in more primitive species such as molluscs (Piferrer *et al.,* 2009).

The first artificial triploid animal produced was the stickleback (Gasterosteidae) in 1959 (Beaumont and Fairbrother, 1991). Research on the occurrence of triploidy in aquatic species commenced in the 1970's and 1980's, and since then a number of aquatic triploid species have been commercially produced. These include the rainbow trout (*Oncorhynchus mykiss*), produced in USA, Canada, France, Japan, UK, Korea, Iran, Poland and Turkey; the brown trout (*Salmo trutta*), produced in UK and France; and Atlantic salmon (*Salmo salar*), produced in Canada and Chile. Other species include chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*), amago salmon (*Oncorhynchus masou*), ayu

(*Plecoglossus altivelis*), cyprind loach (*Misgurnus anguillicaudatus*), and the grass carp (*Ctenopharyngodon idealla*). Shellfish commercially produced include the Pacific oyster (*Crassostrea gigas*), and mussels (*Mytilus edulis*) that have been produced on a semi-commercial scale (Beaumont and Fairbrother, 1991).

Artificial triploidy occurs when the genome of an animal is manipulated to result in the retention of an additional set of chromosomes within the developing embryo. Triploidy can be artificially induced by means of a pressure induction method, chemical treatment, thermal treatment or an electrical field treatment (De Beer, 2004). The rationale for triploid induction is that these animals will partition energy towards growth instead of gonad development and gamete production (Allen *et al.,* 1986; Beaumont and Fairbrother, 1991; Ruiz-Verdugo *et al.,* 2000; Piferrer *et al.,* 2009), resulting in an increased growth rate (Le Curieux-Belfond *et al.,* 2009). Triploids have shown results of growth benefits, and diminished reproductive capacity (Beaumont and Fairbrother, 1991; Cal *et al.,* 2006; Piferrer *et al.,* 2009). However, Prins (2011) found a diminished growth rate in triploid *Haliotis midae* (HM) compared to diploid HM.

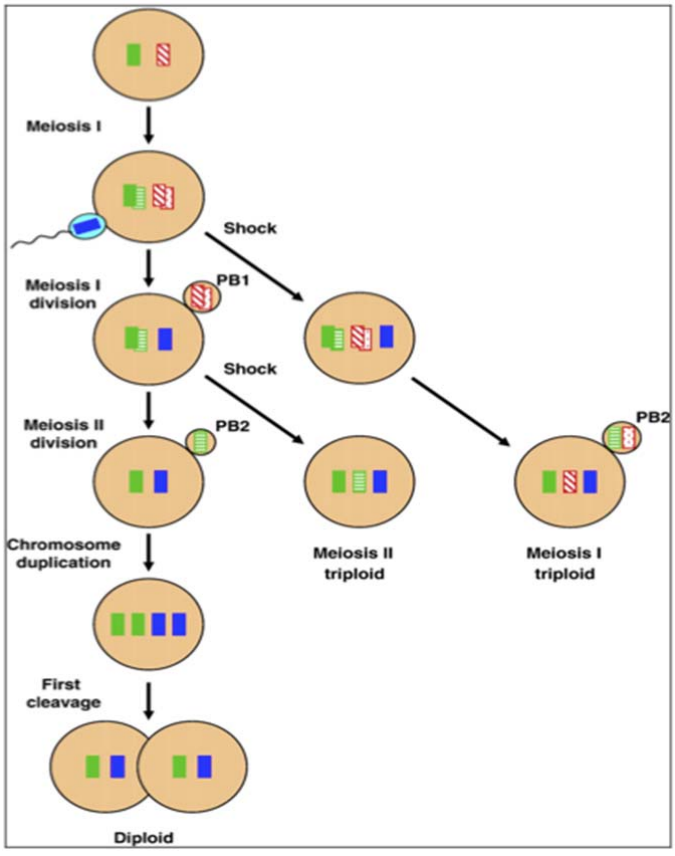



**Figure 2.15.** Sequence of events during triploid induction, by inhibition of the first or second polar bodies (Piferrer *et al.,* 2009).

In marine molluscs, mature ova expelled from the gonad are of Meiosis I stage. Meiosis II is therefore considered a developmental stage, as it only occurs after fertilization (Hahn, 1989; Guo *et al.,* 1992a; De Beer, 2004). For this reason inductions are done on fertilized ova either during meiosis I or meiosis II. By suppressing the formation of the polar bodies (indicated in Figure 2.15), an extra set of chromosomes is

retained within the egg, which can now be considered as being triploid (Gérard, *et al.,* 1999; Piferrer *et al.,* 2009).

Fertility in triploids is said to decrease due to abnormal chromosome pairing among the three sets of chromosomes in the triploid animal (Xie *et al.,* 2008). This causes the formation of imbalanced and non-viable gametes (Xie *et al.,* 2008). Sterility is also said to occur on the basis that the homologous chromosomes in the germ cells of the triploid animal are unable to synapse during meiosis, and therefore normal meiosis is not completed, and gametes will not be formed (Allen *et al.* 1986; Beaumont and Fairbrother, 1991).

There are three main advantages associated with the production of triploid abalone; an increased growth rate resulting from the diversion of energy destined for gametogenesis to somatic growth (Le Curieux-Belfond *et al.,* 2009), a decrease in bad flavouring caused by depleted glycogen levels during spawning, and the decrease in possible negative interactions between farmed and wild abalone (Beaumont and Fairbrother, 1991; Boudry *et al.,* 1998; Rasmussen and Morrissey, 2007; Xie *et al.,* 2008). Other advantages of triploids include increased survival, improved feed conversion ability, and an increased disease resistance (Allen *et al.,* 1986; Cox *et al.,* 1996; Kerby *et al.,* 2002; Piferrer *et al.,* 2009).

Sterility, combined with an increased growth rate, will contain the GMOs as well as decrease the grow-out to harvest time, and allow for more control over the animals (Hu *et al.,* 2007). Cal *et al.* (2006) stated that triploid induction is an effective method to induce sterility although it does not always result in a larger body size. Prins (2011) investigated the growth of triploid HM, and concluded that a decrease in the growth rate of triploid HM was observed. In triploid shellfish, enhanced growth is usually observed, but it is not consistently the case (Beaumont and Fairbrother, 1991; Cal *et al.,* 2006).

*2.6.3.2 Triploid identification*

Slabbert *et al.* (2010) used microsatellite markers as a genetic verification technique for triploid HM. Other methods of triploid verification include DNA karyotyping, genotype satellite DNA markers (Piferrer *et al.,* 2009), chromosome analysis, nuclear sizing, red blood cells, microfluorometry, image analysis, and flow cytometry (De Beer, 2004; Piferrer *et al.,* 2009).

*2.6.3.3 Triploid gonadal development*

Most studies on the effect of triploidy on gonad development indicate varying results, with some cases ranging from normal gametogenic development to true sterility. The degree of arrested development is often found to be dissimilar in male and female animals of the same species (Kudo *et al*., 1994; Brake *et al.,* 2004; Maldonado-Amparo *et al.,* 2004; Li *et al*., 2004; Xie *et al.,* 2008).

Some male fish species show full arrest of gonadal development or spermatogonial proliferation (Allen and Downing, 1986), although sterility is said to be more prevalent in females than in males (Allen and Downing,

1986; Cal *et al.,* 2006; Wong and Van Eenennaam, 2008). According to Arai (2001), triploid induction exerts a more prominent influence on gonadal integrity in female triploid fish. Even though triploidy retards the ovarian growth of the European bass (*Dicentrarchus labrax*; Figure 2.16 and 2.17) to a certain extent, studies have shown evidence of the production of mature oocytes within the gonad (Wong and Van Eenennaam, 2008). It has been noted that in triploid male species, gonadal development is similar to that of their diploid counterparts, with some of the triploid males producing sperm, but with the sperm concentration being lower than that produced by normal diploid males (Cal *et al.,* 2006).

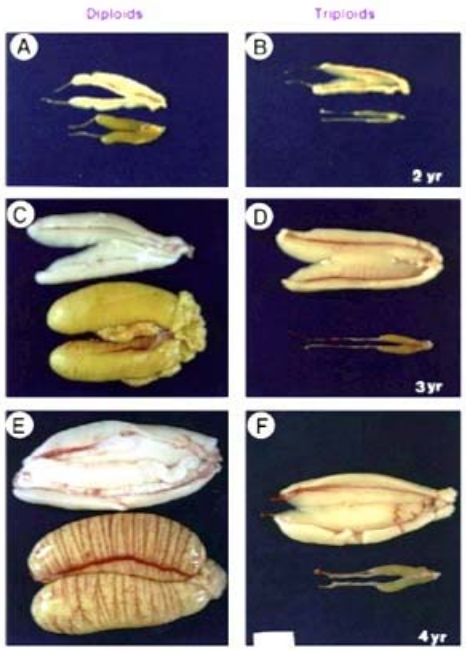



**Figure 2.16.** External view of the European bass (*Dicentrarchus labrax)* gonads (Piferrer *et al.,* 2009).

Zhang *et al. (*2008) found that female gonads of triploid Chinese shrimp (*Fenneropenaeus chinesis*) were completely retarded, with few abnormal spermatids present in the male triploid gonad, Kudo *et al.* (1994) and Li *et al.* (2004) observed that female triploid abalone (*Haliotis diversicolor diversicolor* and *Haliotis discus hannai*) exhibited normal mature oocytes, but no spermatozoa were identified in the male triploids.

Cox *et al.* (1996) conducted an experiment on the triploid rock oyster (*Saccostrea glomerata*), to determine gametogenic development of male and female animals. The triploid female rock oysters (*Saccostrea glomerata*) indicated abnormal gonadal development, where the follicle branching was retarded, and very few mature ova were observed (Cox *et al.,* 1996). The gonad of the male rock oyster (*Saccostrea glomerata*) appeared to arrest gonadal development at the spermatocyte stage, and, where development did occur in the gonad, the spermatozoa were found sparsely within the lumen (Cox *et al.,* 1996). In the thirteen months that the reported trial took place, spawning never occurred. Since the sterilization of animals through triploidy is not 100% effective, it was recommended that physical containment of transgenic animals also be required until further research is completed (Lee and Donaldson, 2001).

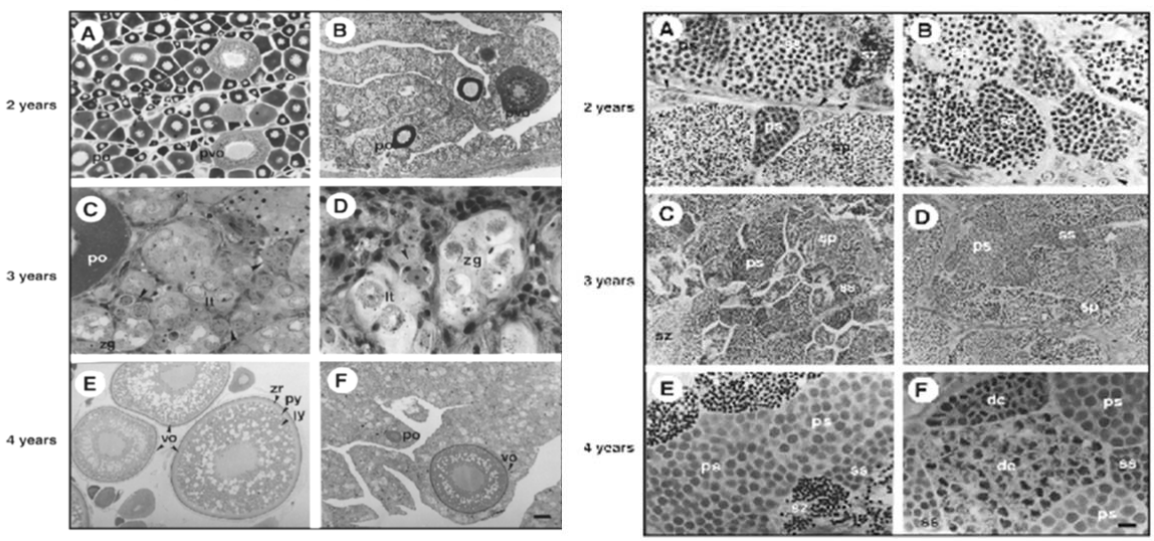



**Figure 2.17.** Histological differences between triploid and diploid female European bass (*Dicentrarchus labrax)* (Piferrer *et al.,* 2009).

*2.6.3.4 Polyploids, Tetraploids and Mosaics*

Polyploid genotypes hold a potential benefit for the aquaculture industry, but have been shown to be lethal in both animals and birds (Rasmussen and Morrissey, 2007). Polyploidy is defined as an organism with additional chromosomes, one additional chromosome would result in a triploid organism, and two additional chromosomes result in a tetraploid organism (Guo and Allen, 1994; Piferrer *et al.,* 2009).

The breeding of a tetraploid organism with a diploid organism would result in a sterile triploid organism, and is the most common method of inducing triploids in the United States and Europe (Piferrer *et al.,* 2009). The breeding of artificially induced tetraploid fish and wild diploid fish would produce sterile triploid progeny (Devlin *et al.*, 2006; Rasmussen and Morrissey, 2007; Wong and Van Eenennaam 2008). The triploid progeny would then not be able to integrate their genetically modified genes into the wild stocks.

Tetraploid induction is a difficult method, and has not been successfully accomplished in other aquaculture species (Piferrer *et al.,* 2009). Tetraploid induction is similar to triploid induction, except the induction happens at a later stage in the embryonic development. Tetraploid fish are produced by inhibiting the cell division of the zygote after the chromosome has been duplicated, which occurs shortly after fertilization as shown in Figure 2.18 (Wong and Van Eenennaam, 2008; Piferrer *et al.,* 2009). Each cell contains four sets of chromosomes, so when diploid ova (produced by tetraploid females) are fertilized by haploid male sperm, the result is a triploid offspring. Diploid sperm that are produced by tetraploids have exhibited reduced fertility, and exhibit difficulty passing through the oocyte micropyle (Piferrer *et al.,* 2009).

Guo and Allen (1994) investigated comparisons between the crossings of tetraploid and diploid pacific oysters (*Crassostrea gigas*) to produce triploids, and inducing triploids using a Cytochalasin B (CB). The treatments resulted in the tetraploid crossed progeny exhibiting fully developed gonads, containing gametes

capable of fertilization, and resulted in a larger degree of triploid gigantism than the triploid pacific oysters (*Crassostrea gigas*) induced using the chemical treatment.

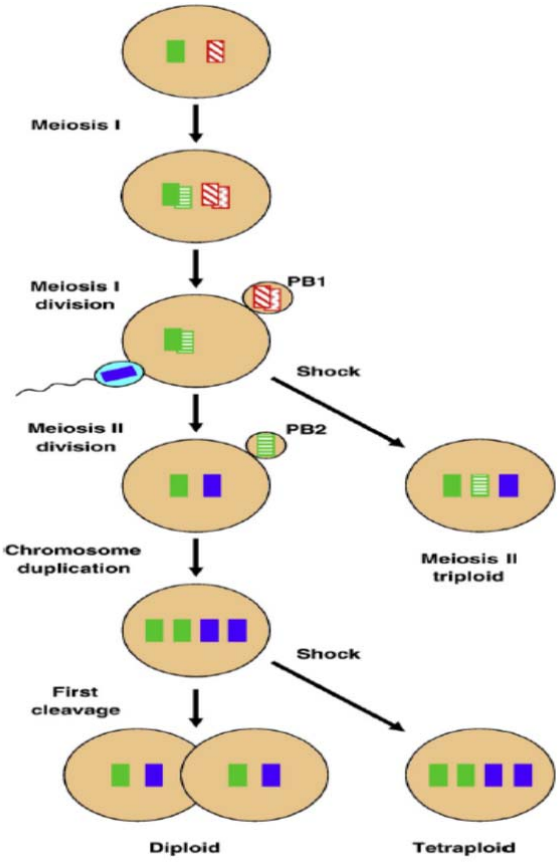



**Figure 2.18.** A diagram representing how tetraploid and triploid induction occurs (Piferrer *et al.,* 2009).

Tetraploidy has only been achieved in rainbow trout (*Oncorhynchus mykiss*), at very low larval survivability, blunt snout bream (*Megalobrama amblycephala*), and mud loach (*Misgurnus anguillicaudatus*). Experiments were conducted on the grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), and yellow perch (*Perca flavescens*), but low yield and survival were also noted (Piferrer *et al.,* 2009). Tetraploid induction is a difficult method and has not been successfully accomplished in other aquaculture species (Piferrer *et al.,* 2009).

The reversal of a triploid organism back into a diploid genotype can occur, and these animals are referred to as heteroploid mosaics (Lee and Donaldson, 2001; Melamed *et al.,* 2002). Heteroploid mosaics contain both triploid and diploid cells, which are thought to have been caused by a chromosome loss. Allen and Guo (1994) documented the occurrence of heteroploid mosaics in triploid Pacific oysters (*Crassostrea gigas*), but no successful reproduction or offspring has been observed. The reversal of tetraploids to diploidy has been observed in salmonids and loach in laboratory experiments (Devlin *et al.,* 2006; Wong and Van Eenennaam, 2008).

## 2.7 Containment strategies

Containment strategies are defined by the conditional weakening of the reproduction and/or viability of life stages that will minimize the effect of transgenic organisms on the surrounding environment (Devlin *et al.,* 2006). Very little information is available on the containment methods in the aquaculture industry. Methods of mechanical containment are however reported in the production of finfish, where net pens and cages form part of the mechanical containment protocols (Daggett *et al.*, 2006; Wong and Van Eenennaam, 2008).

The production of transgenic organisms is not the only concern for necessity of containment methods, as selective breeding, a widely used method to select production animals for specific traits, used on many farms, can similarly cause genetic shifts in wild populations if interactions occur between escaped farmed and wild populations (Devlin *et al.,* 2006). Containment methods should therefore be considered for implementation onto all commercial aquaculture farms even where transgenic organisms are not produced.

Effective containment strategies are part of a crucial approach to the safe application of transgenic fish technology in the aquaculture industry due to uncertainties based on the gene X environment interactions (Devlin *et al.,* 2006). Proposed containment methods to decrease the risk associated with the escape of transgenic organisms include containment by sterility, biological containment, containment by geographical location, thermal containment, chemical containment, and physical containment (Rasmussen and Morrissey, 2007; Wong and Van Eenennaam, 2008). Other risk reduction measures include the use of polyploidy, specifically the use of triploid abalone as they are presumed to be sterile, as well as harvesting of animals before sexual maturity. Although the abovementioned containment methods can possibly decrease the number of farmed and wild interactions, Piferrer *et al.* (2009) stated that only sterile GM fish with no reproductive capability have a future in the aquaculture industry.

### 2.7.1 Biological containment

Sterility as a method of containment is considered the most complete and efficient means of containment (Piferrer *et al.,* 2009). Sterility will prevent any genetic introgression occurring between farmed and wild populations in the case of the escape of GMOs (Allen *et al.,* 1986; Cox *et al.,* 1996; Maclean and Laight, 2000; Cal *et al.,* 2006; Wong and Van Eenennaam, 2008; Piferrer *et al.,* 2009).

Triploid induction is considered as a biological containment method that potentially can reduce the risk associated with interactions between farmed and wild genotypes (Rasmussen and Morrissey, 2007; Wong and Van Eenennaam, 2008). However, as mentioned previously, the induction of triploid species seldom causes 100% sterility. Most studies conducted on the gametogenic development of triploid aquatic species have shown varying degrees of arrested gametogenesis in the triploid species, with some instances of normal gametogenic development, as opposed to true sterility. The arrestation of gonadal development is also found to differ in male and female animals of the same species (Kudo *et al*., 1994; Brake *et al.,* 2004; Li *et al*., 2004; Maldonado-Amparo *et al.,* 2004; Xie *et al.,* 2008).

Other methods that induce sterility may be beneficial as containment methods. Transgenesis that cause gonadal gysgenesis in females, and infertility in males (Maclean and Laight, 2000; Devlin *et al.* 2006), is considered a suitable precaution against the spread of transgenes (Wong and Van Eenennaam, 2008). Other biological containment methods include transgenic sterilization, the artificial inclusion of DNA strands, disruption of embryonic development, and gonad specific transgenes (Maclean and Laight, 2000). Transgenesis is initiated by the antagonism of hormones to disrupt the function of the gonad. Gonadotropin releasing hormone (GnRH) or gonadotropin (GtH) are both necessary for the normal functioning of the gonad, and the antagonism of either or both of these hormones will result in a disrupted gonad function (Maclean and Laight, 2000).

The use of a mono-sex culture, and climatic zones, are proposed biological methods of containment. Mono-sex culture is the use of single sex reared animals. A climatic zone is a concept based on optimal rearing conditions of aquatic species. Animals are reared on land-based systems away from their natural oceanic environments, under their natural environmental conditions, but on escape, they would not survive due to the natural climatic differences (Devlin *et al.,* 2006).

### 2.7.2 Mechanical containment

This type of containment includes a range of physical, mechanical, and closed methods to restrict interactions between farmed fish and the natural environment.

Recirculating systems are the most common method of physical containment in aquaculture. However recirculating systems are used as a sustainable method to reduce water usage, with containment as an advantage. Currently there is a growing interest in the use of recirculating systems, with efficient water and land usage, and the control of the culture environment as its main advantages (Bijo, 2007; Boulet *et al.,* 2010).

Other physical containment methods that have been used in the aquaculture industry include net pens and sea cages (Daggett *et al.*, 2006; Wong and Van Eenennaam, 2008), but these structures are known to fail in bad weather conditions, characterised by presence of predators (Wong and Van Eenennaam, 2008). The British Columbian aquaculture industry estimated that 2% of aquaculture produced Atlantic salmon (*Salmo salar*) escape production pens and cages yearly (McDowell, 2002). Improvements in the physical containment methods have been made, such as tighter nets and stronger net construction, and has seen a decrease in the number of escapees, but a 100% containment has not been achieved (McDowell, 2002).

Mechanical containment methods which have been proposed for the aquaculture industry include micro screens, filter bags, cartridge filters, and sand filters, which are all categorized according to filtration performance and flow rate, and whether they are typically used in seawater units (Colt and Huguenin, 2002). The use of sand filters, which are used to eliminate particulate matter from effluent water from treatment

plants (Palmer, 2010), are primarily based on the ability of the filter to remove faecal matter (Bomo *et al.,* 2003).

Bomo *et al.* (2003) stated that studies on sand filters to remove a specific particle (i.e. pathogens) give an indication of the ability of these filters to remove only the specific particle tested, and the information from the studies cannot be translated into the ability of these systems to remove other types of particles, i.e. life stages of abalone (HM). However, Colt and Huguenin (2002) stated that the size of the sand bed determines the efficacy of the sand filter to filter particles, and not the particles themselves. The use of a coarse sand filter will enable the filtering of larvae and ova and the use of clay allow for much finer filtration. Although the use of sand filters hypothetically will allow for containment of each life stage of abalone according to their size, sand filters can be a source of high labour and high operating costs (Colt and Huguenin 2002; Wong and Van Eenennaam, 2008).

The rate of clogging of sand filters, which is dependent on the volume of water flowing through the sand filters, and the suspended particles within the water, decreases the efficacy of the sand filters. Sand filters can rapidly become dirty affecting the flow of water through the filter (Colt and Huguenin, 2002). These authors also stated that incorporating a sand filter into an existing system that had not been designed for the use of a sand filter could cause incompatibilities within the production system.

Cartridge filters, which are transparent canisters in various sizes, operate optimally at filtration sizes of less than 100 µm (Lenntech, 2013) and therefore would require replacement on a daily basis. The replacement of cartridge filters require turning off the system (Colt and Huguenin, 2002), which is not appropriate for an open continuous flow-through system.

Filter bags are made of polypropylene and available in various pore sizes, are small and can be easily implemented into an existing production system, as they do not require a lot of space. To replace a filter bag only a few seconds of down time is required. Filter bags can also be used repeatedly as they can be cleaned manually (Colt and Huguenin, 2002).

Another proposed type of mechanical containment is geographic isolation where closed recirculating land based systems or bodies of water that are closed off could be used to contain transgenic fish. Often these locations are considered economically non-viable with high operation and capital costs and not necessarily providing 100% contained (Maclean *et al.,* 2002). In France, tetraploid oyster broodstocks are held in closed recirculating systems, which include water treatment systems to prevent spreading gametes or larvae (Piferrer *et al.,* 2009).

### 2.7.3 Chemical containment

Chemical containment methods are based on the principle of exposure of organisms to chemicals. Chemicals are used to induce mortality to the exposed organisms, therefore limiting the interaction of live

GMOs escaping into the natural environment (Wong and Van Eenennaam, 2008). Aquatic species have a narrow tolerance range for survival within their environment and therefore offer limited resistance to exposure to chemicals (Wong and Van Eenennaam, 2008).

Limited information is available on chemical containment methods for aquaculture species, as most chemicals used in the aquaculture industry are administered as antifoulants and disinfectants, or therapeutic agents (i.e. vaccines; Costello *et al.,* 2001). Chemicals are mostly administered in a commercial production system, where it is added to a chamber or sump where the effluent water passes through (Wong and Van Eenennaam, 2008). The effluent water is exposed to the chemical for a specified contact time and is then released into the natural environment. A concern with the application of chemicals in an open flow-through system is the adverse effects on the natural ecosystem (Chen and Liao, 2004). Therefore restoring water to pre-treated quality is common practice to maintain the quality of water flowing back into the surrounding environment (Wong and Van Eenennaam, 2008). These authors also indicated that dissolved chemicals, e.g. bromine, chlorine and ozone, can be administered to kill any transgenic escapees.

*2.7.3.1 Sodium hypochlorite*

Sodium hypochlorite (NaClO) is a chemical compound commonly used as a disinfectant or bleaching agent, used by abalone production systems for disinfection of holding facilities, tanks, and miscellaneous equipment (i.e. buckets, jugs, brooms, brushes, etc.).

The European Union under regulation EEC 793/93 concluded that the use of sodium hypochlorite is safe for the environment (EU, 2007). Due to its high reactivity and instability, sodium hypochlorite quickly disappears from an aquatic environment (EU, 2007).

Hypochlorites are known to decrease in strength over time in storage (Granum and Magnussen, 1987). Temperature, physical energy and light all can break down hypochlorites before it can react with pathogens in water (Granum and Magnussen, 1987). Hypochlorites react with water to form hypochlorous acids which are the disinfecting agent (Granum and Magnussen, 1987). The presence of organic matter (especially protein), and the pH of the water affects the efficacy of hypochlorites (Granum and Magnussen, 1987), as well as temperature, as heating of sodium hypochlorite is said to increase the effectiveness of the chemical (Abou-Rass and Oglesby, 1981).

*2.7.3.2 Chlorine dioxide*

Chlorine dioxide ($ClO_{2)}$ is an environmentally friendly chemical compound disinfection agent that exhibits a wide range of bactericidal and sporicidal activity (Simpson *et al.,* 1993). Chlorine dioxide products come in a liquid, gas or powder form although the gas form is very unstable and easy to volatilize (Pei *et al.,* 2008).

Chlorine dioxide has a long history of being an economical and simple method for disinfection by oxidation (Petrucci and Rossellini, 2005; Vaid *et al.,* 2010). Chlorine dioxide has been used in a number of other

industries, including the dairy industry, beverage industry, the pulp and paper industry, the fruit and vegetable industry, various canning plants, the poultry industry, the beef processing industry, and is seen being used increasingly in municipal potable water treatment facilities (Simpson *et al.,* 1993).

Chlorine dioxide is most effective at higher pH's than other chlorines, and is unaffected by the presence of ammonia (Petrucci and Rossellini, 2005). Chlorine dioxide products come in a liquid, gas or powder form although the chlorine dioxide gas is very unstable and easy to volatilize (Pei *et al.,* 2008). Chlorine dioxide can lose between 7 to 30% of its effectiveness, depending on its concentration and time of exposure to air, due to its high volatility. This means that dilutions prepared for stock solutions cannot be assumed to be quantitative after a certain period of time. (Benarde *et al.,* 1965). The World Health Organization (WHO) has ranked chlorine dioxide as a safe disinfectant, as well as the Food and Drug Administration (FDA), which has allowed the use of aqueous chlorine dioxide in washing fruit and vegetables (Bhunia *et al.,* 2002).

Although chlorine and chlorine dioxide are extremely similar, both being oxidizing agents, chlorine dioxide has 2.5 times the oxidation capacity of chlorine, and has therefore been used in the treatment of water supplies (Bhunia *et al.,* 2002). Chlorine dioxide is said to be less efficient when the organic content of the water is very high, but the advantage over the hypochlorites is that chlorine is retained for longer and so exercise a prolonged bactericidal effect (Rutala and Webber, 2008).

## 2.8 Aim of the study

When national GMO legislation is considered, the Genetically Modified Organisms Act [No. 15 of 1997] of South Africa was formulated to limit the possible harmful effect of GMOs on the natural environment. The Act aims to provide measures for the "…responsible development, production, use and application of genetically modified organisms; to ensure that all activities involving the use of genetically modified organisms (including importation, production, release and distribution) shall be carried out in such a way as to limit possible harmful consequences to the environment…" [sic] (South African Government information, 2013).

The regulations for the contained use of genetically modified plants and animals provide protection for human health and safety (Health and Safety, 2000), and the primary objective of regulations are to ensure any necessary controls for containment are put into place and enforced.

Where the aquaculture industry is considered, there is a lack of clearly formulated containment protocols to manage and contain the potential risk of GMOs, in the case of escape of such animals from commercial production systems. The development of GM aquaculture species to improve, and optimise the cost-efficiency of commercial production systems depends on the formulation and implementation of containment methods, to prevent and minimise the risks associated with GMOs escaping from production systems.

The aim of this study was therefore to investigate the potential of biological, mechanical and chemical methods to contain GMO abalone (*Haliotis midae*) in commercial production systems, and the formulation of guidelines to mitigate the potential impact of GM abalone on the genetic diversity of natural populations around the South African coastline.

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# Chapter 3

# Triploidy as a biological containment method of abalone (*Haliotis midae*)


## Abstract

*Haliotis midae* is an indigenous abalone species occurring along the coast of South Africa and has been commercially farmed since 1990. However, commercial abalone culture hampered by a slow growth rate, and thus long interval to harvest, which negatively affects the cost-efficiency of production systems. Biotechnologies such as genetic modification can potentially be used to enhance growth and thus shorten the interval to harvest. Methods, based on the principle of the partitioning of metabolic energy into growth instead of gonadal development and function, have been developed to induce triploidy in abalone (*Haliotis midae*), with these methods potentially rendering the induced animals sterile. Genetically modified abalone, however, poses a potential ecological risk by having an ability to disrupt the genetic diversity of natural stocks should they escape from commercial production systems. The study therefore investigated the potential of triploid induction as a means of biological containment to prevent, and minimize the potential ecological risk of genetically modified abalone escapees. Gonad tissue samples were collected from 16 triploid male and 15 triploid female abalone, and subjected to histological evaluation to assess the efficacy of the technique to arrest gonadal development, and suppressed the production of viable gametes. The study indicated that triploid induction affected gonadal development and function in both male and female abalone, (*Haliotis midae*). Fertilization trials were performed to assess the fertilizing potential of the triploid gametes. Biopsied sperm obtained from triploid, induced diploid and diploid males were used to conduct the fertilization trials with diploid ova. Because biopsied ova disintegrate when aspirated by means of the biopsy technique, ova were not collected from experimental animals. No larvae were observed from the fertilization trials of triploid male biopsied sperm. However, abnormal larvae (0.01% fertilization) were observed when sperm obtained from the induced diploid animals were used to fertilize diploid ova. The occurrence of abnormal larvae in the fertilization trials of the induced diploid males indicates the possibility that triploid induction by means of the pressure method can lead to the formation of mosaic animals, which indicate that the technique does not render induced animals sterile.


## 3.1 Introduction

Research and development of the abalone, *Haliotis midae* (HM), industry in South Africa is driven by an increase in production efficiency. Research efforts focus on biotechnologies to improve specific production-related traits such as an improved feed conversion rate (FCR), but more specifically in improving the slow growth rate in HM (Devlin *et al.,* 2006*;* Wong and Van Eenennaam, 2008; Visser-Roux, 2011; Arai and Okumura, 2013). Due to many factors a production period of three to five years (Hahn, 1989) is required for animals to reach a harvestable size of at least 70 - 100 grams.

The use of genetically modified organisms (GMOs) to improve production efficiency creates a concern over the possible interaction between farmed and wild organisms, which relates to the potential ability of the genetically modified (GM) animals to outcompete the natural wild stocks through an enhanced fitness, the introduction of diseases, and altering the genetic diversity of wild stocks (Muir and Howard, 1999; Maclean

and Laight, 2000; Hawkins and Jones, 2002; Theil *et al.,* 2004; Devlin *et al.,* 2006; Hu *et al.*, 2007). The probability of selectively bred farmed abalone escaping and settling in the wild is considered as low (Hawkins and Jones 2002; Theil *et al.,* 2004), but this has not been investigated as the industry is relatively young when compared to other animal production industries where GMOs are used as an approach to aid in cost-efficient animal production practices (Theil *et al.,* 2004).

Triploid induction is a biotechnology method that offers a potential opportunity for the manipulation of the growth rate of HM. Triploid induction involves the suppression of the expulsion of the first or second polar bodies during gametogenesis, which results in the production of triploid (3n) abalone that carry an extra set of chromosomes (Boudry *et al.,* 1998). Triploidy can be induced by using hydrostatic pressure, chemical (6-dimethyaminopurine (6-DMAP)), and thermal or electrical field methods (De Beer, 2004; Arai and Okumura, 2013). The key aspect in the induction of triploid animals is that the completion of meiosis I and II only occurs after fertilization in marine molluscs (Guo *et al.,* 1992; Piferrer et al., 2009), which allows for the manipulation of oocytes before fertilization occurs. Triploid animals are believed to divert their energy intended for reproduction into somatic growth (Allen *et al.,* 1986; Beaumont and Fairbrother, 1991; Zhang *et al.,* 1998; Ruiz-Verdugo *et al.,* 2000; Xie *et al.,* 2008; Piferrer *et al.,* 2009), resulting in increased growth rates and true sterility (Allen *et al.,* 1986; Cal *et al.,* 2006; Piferrer *et al.,* 2009).

An improved growth rate will contribute to improving the profitability of the abalone industry, and the global competitiveness of the species, by shortening the interval between hatching and harvest. This will result in lower production costs (Xie *et al.,* 2008). Triploid induction can also be considered as a form of biological containment method that can potentially reduce the risks associated with interactions between farmed and wild genotypes through the suppression of gonad development and function (Rasmussen and Morrissey, 2007; Wong and Van Eenennaam, 2008).

However, most studies conducted on aquatic species on the effect of triploid induction on the gonadal development, however, indicate varying degrees of arrested gametogenesis, with some instances of normal gametogenic development, as opposed to true sterility. The extent to which gonadal development is arrested also differ between male and female animals of the same species (Kudo *et al*., 1994; Brake *et al.,* 2004; Maldonado-Amparo *et al.,* 2004; Li *et al*., 2004; Xie *et al.,* 2008). Zhang *et al.* (2008) found that gonadal development of female triploid Chinese shrimp (*Fenneropenaeus chinesis*) were completely retarded, whilst a number of abnormal spermatids were observed in the gonads of triploid males. Kudo *et al.* (1994) and Li *et al.* (2004) observed that female triploid abalone (*Haliotis diversicolor diversicolor*) and (*Haliotis discus hannai*), exhibited normal mature oocytes, but no spermatozoa were identified in the male triploid gonads.

The development of triploid induction as a method to shorten the interval to harvest in HM has received considerable attention in the last decade. Hydrostatic pressure induction has been the most commonly used method to induce triploidy in HM (De Beer, 2004). Prins (2011) evaluated the growth rate of diploid and triploid HM from the age of 29 to 62 months, and reported a significant reduction in the growth rate of triploid HM, together with a significant reduction in gonad size of the triploid HM. De Beer (2004) and Prins (2011),

however, did not investigate whether triploid induction rendered the induced animals incapable of producing viable gametes that can fertilize diploid HM gametes.

The aim of this study was therefore to determine the influence of triploid induction by hydrostatic pressure on gonad development and function in HM, and to assess the potential of the technique to be used as a form of biological containment of triploid HM.

## 3.2 Materials and methods

This study consisted of two parts, with the first part of the study determining the effect of triploid induction by hydrostatic pressure on gametogenic development in HM, and the second part of the study that determined the fertilizing ability of gametes of triploid HM.

### 3.2.1 Origin of the experimental animals

Experimental work was conducted in a Certified Class 1 GM Laboratory at the HIK Abalone Farm located in Hermanus, South Africa (34°26'1.60"S and 19°13'14.06"E). The triploid gonad removal was conducted in a laboratory at the Mike De Vries Building, Stellenbosch, South Africa (33°55'53.87"S and18°52'1.891"E). Triploid animals were obtained from the I&J Danger Point Abalone Farm near Gansbaai (34°37'45.54"S and 19°17'49.32"E), with the animals originating from a batch of abalone that were induced by means of the hydrostatic pressure method, as described by De Beer (2004). The induced animals were reared under standard commercial husbandry conditions at the I&J facility until the age of 75 months, where after they were collected for the purpose of this study. The ploidy status of the animals were individually verified according to the method described by Slabbert *et al.* (2010), with the technique being able to successfully discern between diploid and triploid animals. Diploid animals approximately 15 years of age (Visser-Roux, 2011) were obtained from the HIK Abalone Farm to be used as a control group in the fertilization trials. Due to the Farm's (HIK) proprietary information, stocking densities, certain time periods and flow rates of housing sections cannot be disclosed.

### 3.2.2 Determining the influence of triploid induction on gonad development

*3.2.2.1 Tagging and sampling of experimental animals*

Prior to the biopsy procedures, all experimental animals were individually tagged by using numbered coloured bee tags as demonstrated in Figure 3.1A, fitted according to the procedure described by Brink *et al.* (2009) and Prins (2011). The tags were affixed to the animals' shells between the respiratory holes and the whorl of the shell (Figure 3.1B) with Superglue (Bostik). Three identical bee tags were attached to each animal as a precaution to ensure that animals can still be identified should a tag be lost due to random factors.

The gender of the animals was determined by visual inspection of gonad colour, i.e. a creamy white indicated a male, and a green colour indicated a female (Visser-Roux, 2011). Individual tissue samples were collected from 100 randomly selected animals (i.e. 50 males and 50 females) with an unknown ploidy status by means of a tentacle biopsy technique, as described by (Slabbert *et al.,* 2010) The samples were placed into labelled Eppendorf tubes (Lasec Laboratories, South Africa) containing 96% ethanol, and packed on ice for transport to the Genetics Laboratory of Stellenbosch University for genetic verification.

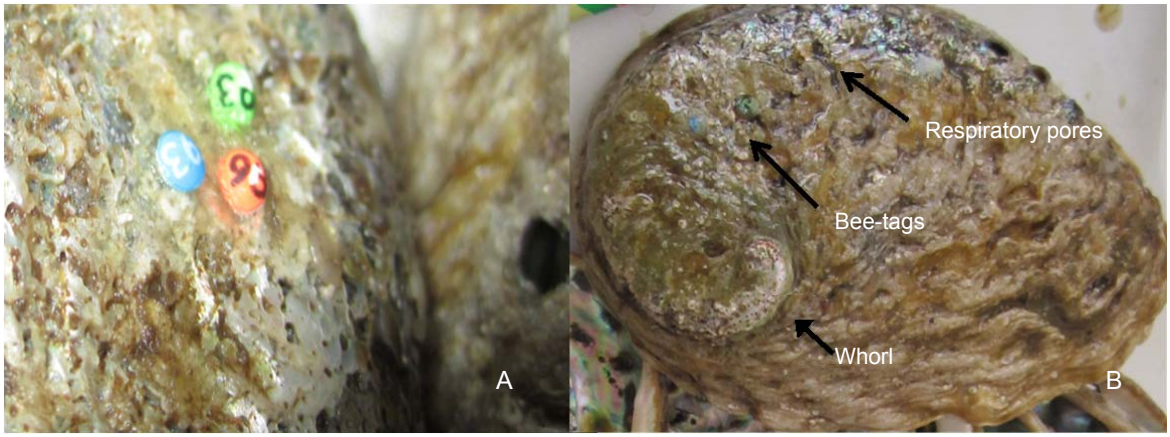


**Figure 3.1.** A: The multi coloured bee-tags. B: Placement of bee-tags on abalone (*Haliotis midae*).

*3.2.2.2 Genetic verification of experimental animals*

Genetic verification of the ploidy status of the sampled animals was performed using microsatellite markers as described by Slabbert *et al.* (2010). From the 100 sampled animals, 43 animals were confirmed as being triploid. Due to tag loss between the time of tissue sample collection, and genetic verification, a total of 31 triploid animals, i.e.16 males and 15 females, were available to obtain gonad tissue samples for histological evaluation.

*3.2.2.3 Preparation and processing of tissue samples for histological evaluation*

After the ploidy status of the sampled animals were confirmed by means of genetic verification, the selected animals were purged overnight in mesh storage bags, and then transported from Danger Point Abalone Farm near Gansbaai to a laboratory in the Mike De Vries building, Stellenbosch. On arrival animals were shucked (i.e. the foot separated from the shell) as described by Mayfield (2010) and Prins (2011), and the entire gonad was removed. The gonads were placed in labelled 50mL Falcon tubes (Lasec Laboratories, South Africa) containing a 10% formalin solution (Visser-Roux, 2011).

Processing of the tissue samples for histological analysis was performed at the Department of Anatomy and Histology of the Tygerberg Campus, Stellenbosch University. Fixed samples were dehydrated with a stepwise series of ethanol solutions of 70% to 100% for 180 minutes each, cleared with xylene for 150 minutes and then fixed in paraffin wax for 120 minutes at 60°C. Sections of the paraffin wax sample were cut to 5 µm and autoclaved for 60 minutes at 60°C. Sections were then rehydrated using a step series of ethanol

of 100% to 70%, and then stained with haematoxylin for four minutes and eosin for 2.5 seconds (HE staining; Visser-Roux, 2011). Once the staining was completed, samples were first dehydrated in an ethanol series of 70% to 100%. Samples were then cleared with xylene for one minute, and mounted onto clean, unused microscope slides (25 X 75 X 1 mm) with a di-n-butyl phthalate in xylene (DPX) mounting medium (Fluka). A cover slip was then placed on top of the tissue sample (Visser-Roux 2011).

*3.2.2.4 Determining the treatment groups*

Initially it was decided only to assess the histology of triploid gonads, and compare the triploid gonads to diploid gonads. When slides of the triploid male and female gonads were assessed, an additional 20 animals, which were at the time considered diploid due to the verification process, were also prepared for histological slides. During the initial histological analysis, it was noted that some of the genetically verified diploid animals (induced diploid) obtained from the I&J facility also exhibited abnormal gonadal development. The farm had advised that the animals in the particular baskets had all gone through a pressure induction by hydrostatic pressure, but it was unclear whether diploid production animals had accidentally been added to the basket or the triploid animals had experienced mosaicasm. Mosaicasm occurs when triploid animals revert to diploid, often having cells of both ploidy types (Piferrer *et al.,* 2009). For this reason the occurrence of mosaicasm in the diploid animals was considered, and for the purpose of the study it was assumed that these diploid animals had been exposed to the hydrostatic pressure induction method. The induced diploid animals were subsequently included in the analysis of data. Histological slides obtained from Visser-Roux (2011) were used as a control group, and the animals were 54 months old.

Animals were classified into three treatment groups, namely:

- A diploid group (ND; control).
- An induced group, verified as diploid (ID).
- An induced group, verified as triploid (IT).

*3.2.2.5 Evaluation of histological preparations*

Gonad histology slides from 71 animals, i.e. 31 triploid (16 males and 15 females), 20 induced diploid (seven females and 13 males), and 20 diploid individuals, were classified by means of histological evaluation to determine the potential of triploid induction to disrupt or arrest gonadal development and function in triploid animals. Histological comparisons of ID and IT animals were assessed using criteria developed by Visser-Roux (2011), to qualify and quantify the development of the triploid gonads (Table 3.1 and Table 3.2).

All slides from treatment groups were analysed at a 4x magnification with an inverted microscope (Olympus IX 51), and photographs were taken with an XC 10 Olympus camera mounted on the microscope (Visser-Roux, 2011).

**Table 3.1.** The nine stages of oogenesis that can be observed in diploid female *Haliotis midae* gonads, as described by Visser-Roux (2011).

| Stage | Description |
|---|---|
| Oogonia | Oogonia are the first developmental stage of mature oocytes, and normally occur attached to the trabeculae. Squamous-shaped follicular cells surround the oogonia and on average have a diameter of 15 to 20 µm. The nucleus of an oocyte is approximately 10 µm in size, and stain light-blue haematoxylin-eosin. The dark blue cytoplasm of oocytes indicates the presence of ribosomes. |
| Stage 1 oocytes | Oogonia develop into Stage 1 oocytes, which are more oval-shaped and still attached to the trabeculae. Stage 1 oocytes range between 20 to 25 µm in diameter, with the nucleus appearing light blue, showing the densely packed chromatin within the nucleus when stained with haematoxylin-eosin. |
| Stage 2 oocytes | The presence of lipid droplets differentiates Stage 2 oocytes from Stage 1 oocytes. Stage 2 oocytes are characteristically 30 to 35 µm in diameter, with a lightly stained nucleus, when stained with haematoxylin-eosin. The average size of the nucleus of Stage 2 oocytes is 20 µm. |
| Stages 3 oocytes | Stage 3 oocytes increase to an average size of 45 to 55 µm, and assume scallop shape. The nucleus appears clear due to uncondensed chromatin. The chorion starts to develop around the oocyte; a higher density of lipid droplets is seen in the cytoplasm. |
| Stage 4 oocytes | Stage 4 oocytes are approximately 60 to 80 µm in diameter, with the nucleolus still staining dark. Lipid droplets within the cytoplasm become more prominent. At this stage of development, the oocytes are still attached at the base to the trabeculae, and the chorion becomes more visible. |
| Stage 5 oocytes | Stage 5 oocytes assume a more columnar shape, ranging from 100 to 150 µm in size. The chorion becomes increasingly visible, and the cytoplasm is characterized by high density of lipid droplets, as well as the presence of yolk platelets. The nucleus appears almost completely transparent, with the nucleolus stain lighter. |
| Stage 6 oocytes | Stage 6 oocytes are elongated, and are attached by means of the cytoplasmic stalk to the trabeculae. The chorion becomes encased within a thick jelly-like coat, which separates cells into loose cell clusters. |
| Stage 7 oocytes | Stage 7 oocytes are now oval in shape, with the cytoplasmic stalk being completely separated from the trabeculae. Stage 7 oocytes lie freely within the lumen, with the chorion and a thick jelly-coat surrounding each cell. |
| Stage 8 oocytes | Stage 8 oocytes are considered to be mature, and ready to be spawned. Mature oocyte size varies between 260 and 300 µm, and the nucleus appears completely clear with nuclear chromatin that is unravelled. Lipid droplets are found in large clusters making the cytoplasm appear clear. |

**Table 3.2.** The five stages of spermatogenesis that can be observed in diploid male *Haliotis midae* gonads, as described by Visser-Roux (2011).

| Stage | Description |
|---|---|
| Spermatogonia | The first developmental stage, and are closely attached to the trabeculae. Spermatogonia are oval-shaped, with an average diameter of 5 to 7 µm. The Spermatogonia nucleus exhibits a clear nucleus with some blue staining which indicates the uncoiled euchromatin. |
| Primary spermatocytes | Occur further away from the germinal epithelium, towards the lumen. Primary spermatocytes are characterized by increasing amounts of dark stained, dense heterochromatin within the nucleus that has an average size of 2.5 µm. Primary spermatocytes have an average diameter of 3 to 5 µm. |
| Secondary spermatocytes | Appear loosely attached to the trabeculae, and are on average 5 µm in length. Secondary spermatocytes stain darker, with a darker stained nucleus than the primary spermatocyte. These cells assume a more oval shape than the primary spermatocytes. |
| Spermatids | Occur in the lumen, and are completely detached form the trabeculae. Spermatids appear oval, and have an average diameter of 2.5 to 3.5 µm. The nucleus show complete condensation of the heterochromatin, appearing very dark after staining. |
| Spermatozoa | The final stage of spermatogenesis and have an average length of 2.5 to 3.5 µm. Spermatozoa, occur within the lumen of the testis, and are considered ready to be released during spawning. |

Once a comprehensive assessment was made, the treatment groups were classified into groups using a scoring method based on work by Cox *et al.* (1996) and Visser-Roux (2011), described in Section 3.2.4.2, to allow for statistical analysis.

### 3.2.3 Determining the fertilizing ability of triploid abalone gametes

*3.2.3.1 Artificial spawning of diploid animals*

Diploid broodstock were induced to spawn according to the sodium hydroxide method as described by Visser-Roux (2011). Sodium hydroxide was added to the tanks to increase the pH of the water; hydrogen peroxide was then added after 15 minutes (Visser-Roux, 2011). Three hours later, residual hydrogen peroxide was removed by means of flushing the system to avoid toxicity (Morse *et al.,* 1977). Tanks were filled with clean filtered seawater, and supplied with aeration. Males began spawning approximately four hours after the induction, and females began spawning six hours after the induction.

The production protocol is to allow fertilization to occur in the female tanks, with after an arbitrary volume of sperm being added to the female tanks after the females have spawned (Paulet, 2011). For the purpose of this study, unfertilized diploid ova were used to assess the ability of biopsied sperm to fertilize diploid ova.

*3.2.3.2 Collection and processing of the diploid gametes*

Sperm and ova collected from 15 diploid males and three diploid females, were pooled for the second part of the study. Sperm were collected from broodstock tanks by means of dipping a 250 mL glass beaker (Lasec Laboratories, South Africa) into the male tanks, and collecting sperm into the beaker (Visser-Roux, 2011). A volume of 1 mL of each sperm sample was transferred from the 250 mL glass beaker into a 1.5 mL Eppendorf tube (Lasec Laboratories, South Africa) using a single channel pipette (Lasec Laboratories, South Africa).

A measure of 1 µL formaldehyde (40%; Protea Chemicals, South Africa) was added to the same Eppendorf tube, using a single channel pipette. The addition of formaldehyde slows the swimming action of the sperm. A cover slip (1.5 x 1.5 cm; Lasec Laboratories, South Africa) was carefully placed over a Neubauer haemocytometer before the sperm were loaded into the Neubauer haemocytometer. An aliquot of 15 µL of sperm was then loaded into the Neubauer haemocytometer using a single channel pipette. Care was taken to avoid the formation of air bubbles when loading samples into the Neubauer haemocytometer chamber.

The concentration of sperm was calculated by counting the number of sperm within five blocks on each side (total of 10 blocks) of the Neubauer haemocytometer (Hafez and Hafez, 2000). The average of sperm per block was calculated. By multiplying the average number of sperm per block with 50 000, results in the number of sperm in 1 mL of the 250 mL water sample collected from the male tanks. An equation ($C_1V_1 = C_2V_2$) was used to calculate the volume of the sample water that would contain 50 000 sperm (Visser-Roux, 2011).

After females have been induced to spawn, the air- and water flow was turned off allowing the negatively buoyant unfertilized diploid ova to settle on the bottom of the tank. A serological 10 mL plastic pipette (Lasec Laboratories, South Africa) was used to draw ova from the bottom of the tank and transferred to a 250 mL Erlenmeyer glass beaker (Lasec Laboratories, South Africa). The ova concentrations were calculated volumetrically by counting the number of ova per 1 mL using a microscope (Visser-Roux, 2011). The counts were repeated three times to verify the average number of ova per mL. The volume of sample water containing 1000 ova was calculated by using the ova/mL calculated.

*3.2.3.3 Triploid and induced diploid gamete collection using a biopsy method*

Five male (two TT and three TD) animals were transported from I&J, Gansbaai and used to conduct the fertilization trials. The ploidy status of the triploid and induced diploid animals was verified in the same way as described in Section 3.2.2.2.

Prins (2011) found that even after a prolonged conditioning period, triploid individuals did not spawn following induction, therefore for this study triploid gametes were obtained using the biopsy technique (Figure 3.2) described by Visser-Roux (2011).

**Figure 3.2.** The positioning of the needle in the gonad during a biopsy technique to collect sperm from an adult male abalone (*Haliotis midae*).

Sperm were extracted from triploid males using a 26-gauge needle. The needle was filled with 15 µL of filtered seawater, gently inserted into the gonad, after which seawater is injected allowing the loosening of the gametes in the lumen. Using the same needle, sperm were then extracted from the gonad, and transferred to 10 mL Nunc wells (Lasec Laboratories, South Africa) containing filtered seawater. Male gametes are visible as a creamy/white clump of tissue. Biopsied ova and sperm tend to remain bound by epithelial tissue, causing sperm to appear coagulated, rendering them in some instances, immobile.

*3.2.3.4 Evaluation of the fertilizing potential of triploid and induced diploid sperm*

Fertilization experiments were conducted with gametes obtained from all three treatment groups shown in Table 3.3. Because biopsied ova disintegrate when aspirated by means of the biopsy technique, ova were not collected from triploid (IT) or induced diploid (ID) female animals (Visser-Roux (2011).

**Table 3.3.** A representation of the three treatment groups of the sperm and ova combinations, to determine the fertilizing ability of triploid, diploid and induced diploid abalone (*Haliotis midae*).

| **Treatment** | **Ova/Sperm combination** | |
|---|---|---|
| | **Ova** | **Sperm** |
| Treatment one (control) | Diploid Spawned (ND) | Diploid Spawned (ND) |
| | Diploid Spawned (ND) | Diploid Biopsied (ND) |
| Treatment two | Diploid Spawned (ND) | Biopsied 1 (ID) |
| | Diploid Spawned (ND) | Biopsied 2 (ID) |
| | Diploid Spawned (ND) | Biopsied 3 (ID) |
| Treatment three | Diploid Spawned (ND) | Triploid biopsied 1 (IT) |
| | Diploid Spawned (ND) | Triploid biopsied 2 (IT) |

Due to the coagulated appearance of extracted sperm, the concentration of the extracted male induced diploid and male triploid sperm could not be determined. Sperm were extracted by means of the biopsy

method as described in Section 3.2.3.3, from each of the male (ID and IT) animals. Once sperm had been extracted, fertilizations were performed. Extracted sperm (contents of the 26-guage needle) from each male was added to separate Nunc Wells (Lasec Laboratories, South Africa), prefilled with 10 mL of filtered seawater. A volume of collected ova (collection and concentration calculation described in Section 3.2.3.2) containing 1 000 spawned diploid ova was added to the same Nunc wells using a single channel pipette (Lasec Laboratories, South Africa). Nunc wells were incubated (LM 570-R, MRC) at 18°C for 18 hours for fertilization to take place. The procedure of carrying out fertilizations were the same for all treatments (Table 3.3), and each treatment was repeated three times. The trials were done on farm (HIK) and the available systems did not allow for the extended observation of larvae over the five day metamorphosing period. Therefore only the hatch out rate was observed, which occurs 16 - 18 hours post fertilization.

### 3.2.4 Data recorded for the histological gonad development analysis of triploid abalone (*Haliotis midae*)

*3.2.4.1 Relative age versus maturity*

The stages of gonadal development exhibited by HM between ages one and four, was described by Visser-Roux (2011). Experimental triploid and induced diploid males and females were scored between one and four according to the criteria in Table 3.4 as defined by Visser-Roux (2011).

**Table 3.4.** The gametogenic development observed by Visser-Roux (2011) in abalone *Haliotis midae* gonads of animals aged one to four years.

| Age | Description of gametogenesis development |
|---|---|
| One | A definite gonad cavity is observed with progenitor germ cells developing from the epithelium into the gonad cavity. Squamous germ cells are visible along the germinal epithelium, but can not be identified. |
| Two | All nines stages of oogenesis are visible, specifically stage 2 and 3 oocytes. |
| Three | A number of stage 1 to 3 oocytes are visible, but stage 8 oocytes are more predominant, indicating a readiness of females to spawn. |
| Four | A large number of stage 2 and 3 oocytes, with stage 7 and 8 oocytes being more prominent than in the younger age groups. Jelly coats surrounding stage 7 and 8 oocytes appeared thick, and separated the oocytes; oocytes are compressed and distorted as the gonad increases. |

The histological preparation from each animal was observed, and scored on the scale depending on the age at which similar gonadal development was observed in diploid of the same gender, i.e. if the animal showed similar gonad development to a one-year-old diploid animal as described by Visser-Roux (2011), it would be scored as a “one”.

*3.2.4.2 Classification of the gonad development of triploid abalone (*Haliotis midae*)*

In order to evaluate the gametogenic development of the male and female triploid animals, a grading scale was developed according to the absence and/or presence of immature and mature gametes. This scale was based on the classification system of oogenesis and spermatogenesis (Table 3.5) as devised by Visser-Roux (2011), as well as Cox *et al.* (1996).

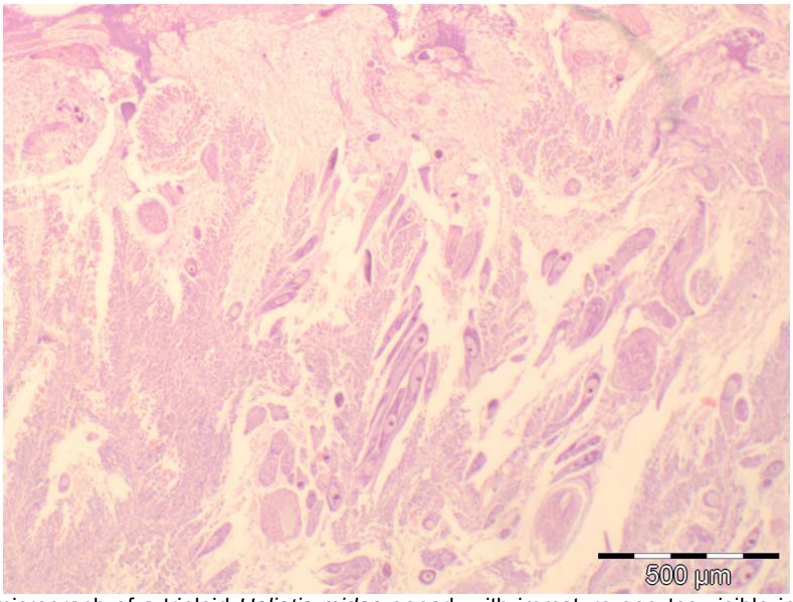


**Figure 3.3.** A micrograph of a triploid *Haliotis midae* gonad, with immature oocytes visible in an ovarian compartment (4X magnification).

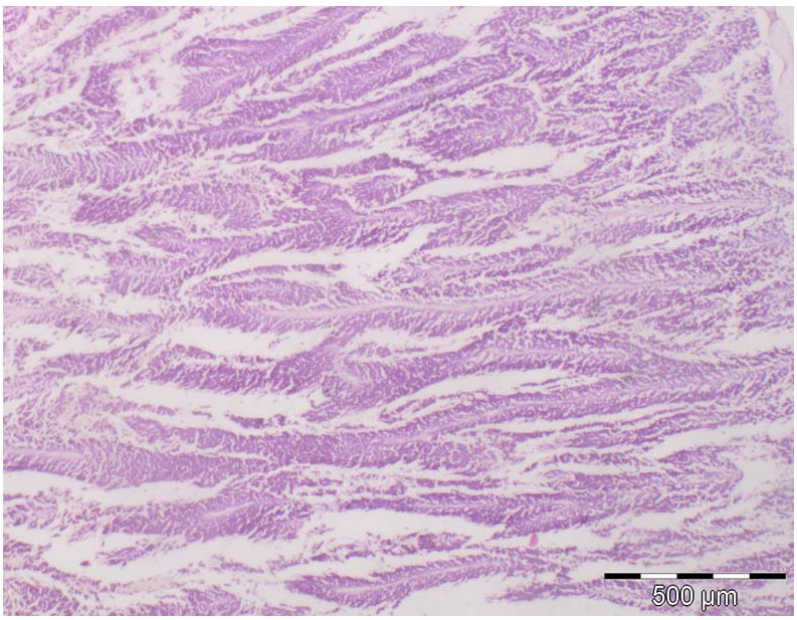


**Figure 3.4** A micrograph of a triploid *Haliotis midae* gonad, depicting an empty lumen (no spermatozoa (magnification 4x)).

**Table 3.5.** A classification system for the evaluation of the effect of triploid induction on the function and integrity of the gonads of triploid male and female abalone (*Haliotis midae*).

| Group | Description |
|---|---|
| One | A gonad characterized by the presence of a small number of immature, and a lack of mature gametes. |
| Two | A gonad characterized by a presence of both mature and immature gametes. |
| Three | A gonad characterized by the presence of mature gametes, and a lack of immature gametes. This is typically seen in ripe animal ready to spawn. |

Triploid male and female animals were classified into one of the three groups (Table 3.5), depending on the absence or presence of gametes in the gonads (See Figure 3.3 and Figure 3.4).

*3.2.4.3 Differences in oogenesis between treatment groups*

The classification system of oogenesis (Table 3.1) in HM developed by Visser-Roux (2011) was used to record the number of each oocyte stage observed in individual treatment animals (ID, IT and ND). The five stages of spermatogenesis of male triploid animals, was, however, not evaluated, as the high sperm density complicated the determination of the spermatogenesis stages.

### 3.2.5 Data recorded for the fertilization analysis of triploid and induced diploid abalone (*Haliotis midae*)

After the incubation period of 16 hours post fertilization, the Nunc wells were removed from the incubator, and the contents of each Nunc well was then transferred with a 10 mL syringe (Lasec Laboratories, South Africa) into individual Petri dishes (Lasec Laboratories, South Africa). Care was taken to acquire all the biological content of the Nunc well into the syringe The Petri dishes were then examined under an inverted microscope (Olympus IX 51) at 4x magnification.

The following data was recorded for each treatment group (Figure 3.5):

- The number of fertilizations observed, which included cleaving of the ova.
- The number of larvae observed.
- The number of abnormal larvae observed.
- The number of unfertilized ova observed.

The number observed of each of the abovementioned treatment groups were counted and recorded. Percentages (%) were for calculated for each of the above-mentioned data groups per treatment (Table 3.3), in order to determine their fertilizing ability. These percentages (%) were statistically analysed.

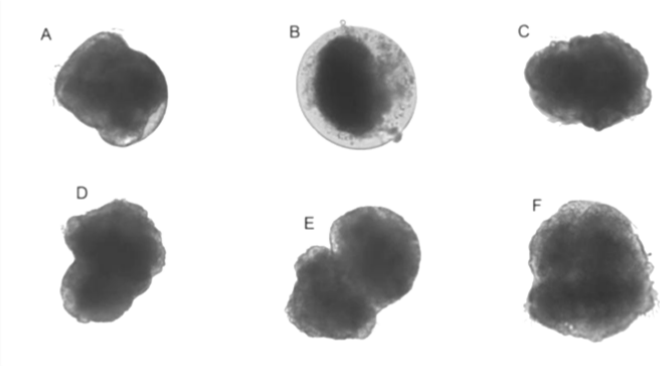


**Figure 3.5** Larval abnormalities in *Haliotis midae* (A= normal larvae, B=larval in arrested development; C – F = morphological abnormalities) (Visser-Roux, 2011).

### 3.2.6 Statistical analysis

All statistical analyses were done using SAS for Windows version 9.3 (2011). Gonadal development data was analysed through an Analysis of Covariance method, using PROC GLM. The relative age versus maturity trait was included as a co-variable in the statistical analysis of the male and female gonad data. Contingency tables Chi-square test was used to determine the significance of difference in expected frequencies of triploid and induced diploid female abalone compared to the observed frequencies of the diploid animals. Mixed Model (PROC mixed) analysis was used to determine fertilization success of triploid and induced diploid abalone. In all cases, Least Square Means (LSM) and Standard Error of the Mean (SEM) were calculated to interpret the results due to the unbalanced number of observations in treatment groups.

## 3.3 Results

### 3.3.1 Histological evaluation of triploid, induced diploid and diploid abalone (*Haliotis midae*) gonadal samples

The degree of gametogenic development observed in the triploid and induced diploid groups differed significantly from the diploid control group ($P < 0.05$; Table 3.6). The results indicated that the treatment groups differed in terms of scoring, according to the scale (Table 3.5). Diploid females obtained a mean score of 2.43 ± 0.31 (Table 3.6), followed by limited degrees of gametogenic activity of the induced diploid females and triploid females obtaining mean scores of 1.80 ± 0.38 and 1.47 ± 0.46, respectively (Table 3.6).

**Table 3.6.** The degree of gametogenic development (mean score ± SEM) observed in diploid, induced diploid, and triploid male and female abalone (*Haliotis midae*), aged 75 months.

| | Treatment (number of animals) | Gametogenic development Score | SEM |
|---|---|---|---|
| **Female** | Diploid (8) | 2.43[a] | 0.31 |
| | Induced diploid (7) | 1.80[b] | 0.38 |
| | Triploid (15) | 1.47[b] | 0.46 |
| | | | |
| **Male** | Diploid (12) | 2.97[a] | 0.29 |
| | Induced diploid (13) | 2.30[b] | 0.49 |
| | Triploid (16) | 1.17[c] | 0.42 |

[a, b, c] Rows with different superscripts differ significantly (P < 0.05)

Significant differences were observed between the gametogenic development of all three male HM treatment groups. Diploid males exhibited the highest degree of gametogenic development with a mean score of 2.97 ± 0.29 (P < 0.05; Table 3.6). Induced diploid males exhibited a mean score of 2.30 ± 0.49, and triploid males showed the least developed gonads, with a mean score of 1.17 ± 0.42.

### 3.3.2 A comparison of the degree of oogenesis between triploid, induced diploid and diploid abalone (*Haliotis midae*)

The number of oocytes observed within the gonads of triploid, induced diploid and diploid females differed significantly (P < 0.05). Figure 3.6 depicts the frequencies of the number of gametes (per treatment group), of each of the nine stages of oogenesis observed in the gonads.

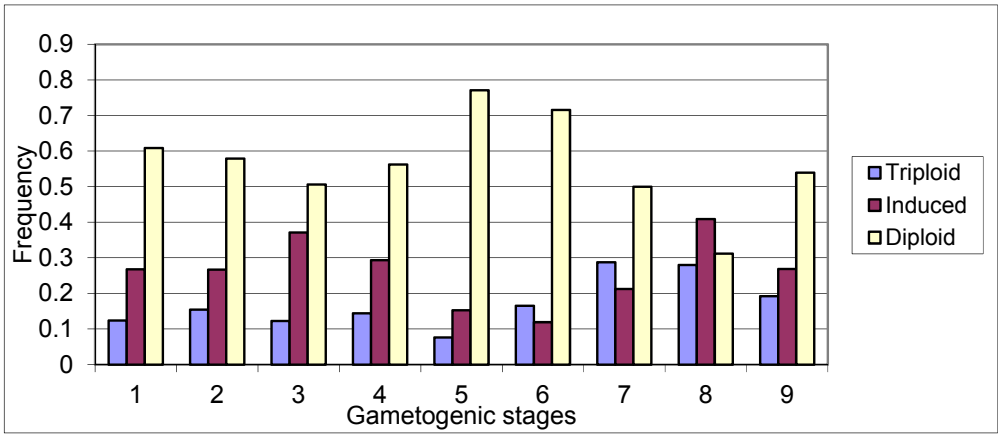


**Figure 3.6**. A comparison of the extent of oogenesis (i.e. frequencies of gametes observed per stage of oogenesis) in diploid, induced diploid, and triploid abalone (*Haliotis midae*), at an age of 75 months.

Three oogenesis stages i.e. stages one, five and six indicated the largest difference in the frequency of oocytes observed between triploid and diploid female animals.

### 3.3.3 The Fertilizing ability of triploid and induced diploid abalone (*Haliotis midae*)

The fertilizing ability of the treatment groups (triploid and induced diploid) differed significantly from the fertilizing ability of the diploid control group ($P < 0.05$; Table 3.7).

A fertilization rate of 0.01% (abnormal larvae) occurred in the treatment with spawned diploid ova fertilized with induced diploid biopsied sperm (Table 3.7), but due to its very small value it did not differ significantly from those where no fertilizations were observed. Fertilization did not take place in treatments of spawned diploid ova fertilized with triploid male biopsied sperm.

**Table 3.7.** The comparative Least Square Means (LSM) and standard error of the mean (SEM) for the percentage (%) fertilization of four treatment groups, using a combination of ova from diploid spawned abalone and sperm from triploid, diploid and induced diploid abalone (*Haliotis midae*).

| Treatment | | Fertilization rate | SEM |
|---|---|---|---|
| **Ova** | **Sperm** | **%** | |
| Diploid Spawned | Diploid Spawned | $58.3^{a}$ | 5.61 |
| Diploid Spawned | Diploid Biopsied | $11.7^{b}$ | 5.61 |
| Diploid Spawned | Induced Diploid Biopsied 1 | $-1.08E^{-15b}$ | 5.61 |
| Diploid Spawned | Induced Diploid Biopsied 2 | $-1.08E^{-15b}$ | 5.61 |
| Diploid Spawned | Induced Diploid Biopsied 3 | $2.96E^{-15b}$ | 6.87 |
| Diploid Spawned | Triploid Biopsied 1 | $-1.08E^{-15b}$ | 5.61 |
| Diploid Spawned | Triploid Biopsied 2 | $-1.08E^{-15b}$ | 5.61 |

$^{a, b, c}$ Rows with different superscripts differ significantly ($P < 0.05$)

A 58.3 ± 5.61% fertilization rate (Table 3.7) was observed when spawned diploid ova fertilized with spawned diploid sperm, and a fertilization rate of 11.7 ± 5.61% occurred when diploid spawned ova were fertilized with biopsied sperm (Table 3.7).

## 3.4 Discussion

Genetically modified organisms (GMOs) such as triploid abalone are created with the intention of improving the production efficiency of commercial production systems through the improvement of e.g. growth rates and feed conversion ability, which in turn can contribute to the cost-efficient management of abalone production systems. Triploid abalone are believed to be sterile (Allen *et al.,* 1986; Cal *et al.,* 2006; Piferrer *et al.*, 2009), with proposed advantages being reflected in improved growth rate and product yield, mainly due to the diversion of gonad maturation energy into somatic growth, and mitigation of the potential effect of escapees on the surrounding wild population and species biodiversity (Allen and Downing, 1986; Beaumont and Kelly, 1989; Liu *et al.,* 2004; Piferrer *et al.,* 2009; Prins, 2011).

Triploid induction methods were developed for *Haliotis midae* (HM), using hydrostatic pressure (De Beer, 2004). Prins (2011) found a significant reduction in the size of triploid HM gonads, when compared to diploid gonads, but no studies have yet investigated whether inducted HM are rendered sterile. Results from this study indicated that the induction of triploidy was not effective in completely suppressing gametogenic development. Triploid and induced diploid HM exhibited gonadal growth, as well as produced gametes. Triploid induction, did however, impact negatively on male and female HM gonadal development. The histological comparisons of gametogenesis development in diploid and triploid HM of both genders indicated significant differences in their development. Triploid female HM gonads exhibited a mean score of 1.47 (characterized by a small number of mature gametes mixed with immature gametes), compared to the diploid HM gonads with a mean score of 2.43, where mature ova were observed. Male triploid HM gonads also exhibited limited gonadal development with a mean score of 1.17 (characterized by a small number of immature gametes, and a complete lack of mature gametes), compared to diploid male HM gonads, which showed a mean score of 2.97. Visser-Roux (2011) indicated that diploid HM aged 54 months should exhibit full sexual maturity with a lumen full of mature gametes. However, the gonadal development results indicated that male and female diploid HM exhibited a few immature gametes within their gonads (with mean scores of 2.43 and 2.97 respectively); this could be due to the large variation noted within the HM species (Jansen, 2011).

The abnormal gonadal development observed in triploid male and female HM is similarly seen in other triploid molluscs (Xie *et al.,* 2008). The triploid Mediterranean mussel (*Mytilus galloprovincialis*), and the lion-paw scallop (*Nodipecten subnodosus*), were found to go through a degree of gametogenesis, but the development remained arrested when compared to the gametogenic development of the diploid varieties (Brake *et al.,* 2004; Maldonado-Amparo *et al.,* 2004).

Zhang *et al. (*2008) found that in triploid Chinese shrimp (*Fenneropenaeus chinesis*) that gametogenesis in females was completely supressed, but few abnormal spermatids were present in gonads of male triploids. The findings for the male triploid Chinese shrimp (*chinesis*), are similar to the results obtained for the triploid male HM gonad, but findings are not similar to the results obtained for the female triploid HM gonad, where gametes were observed and gametogenic development was not completely suppressed as seen in the triploid female Chinese shrimp (*chinesis*).

Kudo *et al.* (1994) and Li *et al.* (2004) observed that female triploid abalone (*Haliotis diversicolor diversicolor*) and female triploid abalone (*Haliotis discus hannai*) developed normal mature oocytes, but no spermatozoa were identified in the male triploids. These findings are similar to the gametogenic development observed in the triploid HM, where a number of mature gametes were observed in the triploid HM female, but male triploid HM developed no spermatozoa. Contradictory results have been reported for the triploid surf clam (*Spisula solida*), *and* Japanese pearl oyster (*Pinctada fucata*), in which normal gametogenic development was achieved (Brake *et al.,* 2004).

Spermatogenesis was arrested in triploid and induced diploid male HM. The developmental stages of the male HM were not statistically analysed, as the determination of the number of sperm in a diploid HM lumen proved difficult. Histological evaluation indicated that spermatogenesis progressed up to the primary spermatocyte stage in the triploid males. Thorgaard (1983) and Zhang *et al.* (2008) observed similarities within the gonad of the triploid male Pacific oyster (*Crassostrea gigas*), where the triploid male oyster exhibited typical development until the spermatocyte stage, after which further development was arrested. Ibarra *et al.* (2004) consistently found that development in the male triploid lion-paw scallop (*Nodipecten subnodosus*) showed gametogenic development until the spermatocyte stage, after which further development was arrested. Arrested gametogenic development has also been observed in triploid Sydney rock oysters (*Saccostrea glomerata*) and the European bass (*Dicentrarchus labrax*) (Cox *et al*., 1996).

Oogenesis was arrested in triploid and induced diploid female HM. The development of oogenesis in female triploid HM exhibited lower numbers of gametes per stage than diploid female HM.

Although triploid females exhibited the least number of gametes overall per gonad, the large variation observed within treatment groups in relation to the number of gametes complicated the quantification of stage of development. Two oogenesis stages, i.e. stages five and six showed the largest difference in number of oocytes between triploid and diploid female HM. The triploid female HM gonads did however display similar number of gametes to the diploid female animals for stage 8 oocytes. It could be hypothesized that the large differences observed in stage five, are attributable to the induction method affecting the endocrine system of the female abalone. Stage five is characterized by the formation of the chorion around the ova, and the formation of yolk platelets within the ova for larval survival. If the development of the oocyte does not occur correctly, the mature ova may not be contain sufficient nutrients for the development of a larva, as an ovum with high protein, lipid and carbohydrate content will supply in the developmental needs of a larva (Sangsawangchote *et al.,* 2010). The abnormal development of the oocytes during Stages eight and nine, which is considered the final maturation stages, can potentially impact negatively on the viability of HM ova. The possible effect of triploid induction on the endocrine system of HM pertaining to the disruption of hormones associated with gonad development should therefore be further investigated.

Fertilization trials were performed in order to evaluate the fertilizing ability of triploid male HM gametes. Fertilizations were not performed using female HM biopsied ova, as they are bound by epithelial tissue, and tend to disintegrate when removed from the gonad using the biopsy technique (Visser-Roux 2011).

In treatments where triploid HM sperm fertilized diploid HM ova, no larvae were obtained. A fertilization rate of 0.01% (abnormal larvae) was observed in the fertilizations of induced diploid HM, but due to its very small value it did not differ significantly from those where no fertilizations were observed. However, the larvae observed were abnormal, and no viable larvae were observed.

The extraction of sperm by means of the biopsy technique can potentially affect the outcome of fertilizing ability of the HM sperm due to the coagulation of tissue, although fertilizations (11.7%) were observed with biopsied diploid sperm obtained from the control group, which indicates that biopsied HM sperm are able to fertilize diploid HM ova. The fact that triploid HM males exhibited a mean developmental score of 1.17, characterizing the gonad as having only a small number of immature gametes, and a complete lack of mature gametes supports the result that triploid male HM produce sperm that were incapable of fertilizing diploid HM ova. Beaumont *et al*. (2009) found that triploid Atlantic salmon (*Salmo salar*) sperm are able to activate ova, but were not able to fertilize the ova.

The fact that a 0.01% abnormal fertilization was obtained when diploid HM ova were fertilized with induced diploid HM sperm, could indicate the occurrence of possible mosaics within the treatment group. A study conducted on triploid oysters (*Crassostrea gigas*) reported on the occurrence of mosaics, however the development of sexual maturation in the mosaic oysters could not be proved (Allen and Guo, 1996). The method of induction to produce triploid animals may have varying effects on the outcome of triploid and mosaic animals and the development of their gonads. The potential of the four methods of triploid induction should be investigated to compare the gametogenic development between induction methods as well as assess the number of mosaics that occur using each method.

## 3.5 Conclusion

The objective of this study was to determine the influence of triploid induction on gonad development and function in *Haliotis midae* (HM), and evaluate the use of triploidy as a biological containment method. The results indicated that triploid induction by hydrostatic pressure treatment negatively affected the degree of gametogenic development in triploid male and female HM. Complete sterility which is required for triploidy to be considered as a reliable method of biological containment of GMO material in commercial systems could, however, not be confirmed due to the presence of mature gametes observed in triploid male and female gonads.

Although this study showed that abnormal gonad and gamete development occurred in triploid HM, and no fertilizations were observed, the sample sizes available for the study were small. Further research using larger sample sizes is necessary, particularly to determine the fertilizing ability of triploid males as well as a method of obtain triploid ova in order to assess their fertilizing ability.

The concern of potential escape or release of gametes and/or animals from productions systems necessitates the development of effective containment protocols. This study highlighted the need to refine triploid induction in HM to ensure that the technique can indeed be propagated as a form of biological containment of GM abalone. Special attention should be given to how triploid induction interfere with the normal endocrine regulation and control of gametogenesis and spawning in induced HM individuals.

The occurrence of mosaics in this study indicated that the reproductive potential of the induced animals were negatively affected, but the extent of disruption of gonadal development cannot be considered an effective means of containment.

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# Chapter 4

# Mechanical containment of abalone (*Haliotis midae*)


## Abstract

The development of genetically modified aquatic organisms presents an opportunity to improve the efficiency of aquaculture production. The genetic modification of abalone, (*Haliotis midae*), to improve its growth rate is of particular interest, for it can potentially decrease the interval from hatch to market. A concern in the production of genetically modified abalone, however, is their ability to escape from production systems, and potential impact on the natural environment. In South Africa, commercial abalone operations use an open continuous flow-through systems, offering opportunity for potential genetically modified escapees to be introduced to natural abalone populations. Life stages of abalone (i.e. spermatozoa, ova, larvae, spat, immature and mature abalone), due to size and behaviour differences, require specific containment strategies to ensure effective containment of genetically modified abalone in such systems. This study therefore investigated the use of 100 µm polypropylene filter bags, and three materials (AstroTurf, carpet and Velcro) to potentially contain the life stages of abalone in an open continuous flow-through system. The use of a 100 µm polypropylene filter bags was not effective in containing female gametes in the broodstock section system. Sperm were not used in the treatment of polypropylene filter bags as an appropriate size filter bag was not available for the size of sperm. Polypropylene bags, with a pore size of 100 µm however, resulted in effective containment of one-day-old larvae and settlement larvae, although the results did not differ significantly from the current on-farm method of containment (120 µm banjoe sieve) already in place in the larvae section. The use of the obstructive materials did not show significant differences in the ability to contain spat over a 48-hour period, compared to the control treatment. Further research should investigate the use of obstructive materials over a longer time period, as the temperature of seawater effects the animals escapism. Future research on economical, effective usage of space, and low maintenance methods of mechanical containment that can be implemented into existing commercial production systems should be focused on.


## 4.1 Introduction

*Haliotis midae* (HM) is the only abalone species that is commercially produced in South Africa, with a total of 12 abalone farms located along the South African coastline (DAFF, 2012). Abalone take up to five years to reach a marketable size of 70 - 100 grams (Hahn, 1989), and therefore it is important that farming systems are managed optimally to ensure that the production of marketable animals is as cost-efficient as possible (Duame, 2003).

In aquaculture activities, biotechnological manipulations used to enhance production performance include gene transfection technologies, as well as the induction of polyploidy (Galli, 2002; De Beer, 2004). In abalone, triploid induction, a form of polyploidy induction, is considered as a method to induce sterility, resulting in an improved growth rate, and more efficient production through a decreased use of feed over time, and a decreased holding time in production units (Allen and Downing, 1986; De Beer, 2004; Cal *et al.,* 2006; Piferrer *et al.,* 2009). The increased growth rate linked to sterility is said to occur as sterile triploid animals partition energy and protein into growth, rather than the development and maintenance of gonadal

function (Allen and Downing, 1986; Beaumont and Fairbrother, 1991; Zhang *et al.,* 1998; De Beer, 2004; Liu *et al.,* 2004).

A risk associated with the production of genetically modified organisms (GMOs), is the exposure of naturally occurring organisms in the surrounding environment to the escaped GMOs (Muir and Howard, 1999; Maclean and Laight, 2000; Beardmore and Porter, 2003; Hu *et al.,* 2007). Exposure of wild stocks to genetically modified (GM) abalone may compromise the genetic integrity of the wild stocks through advantages related to genetic modification, which include increased reproduction ability, increased disease resistance and increased predation ability (Muir and Howard, 1999; Maclean and Laight, 2000; Hawkins and Jones, 2002). The risk of GMOs interacting with the environment and wild stocks can be minimized or prevented through effective containment methods (Rasmussen and Morrissey, 2007; Wong and Van Eenennaam, 2008). Three types of containment methods are available namely, biological (complete sterility), mechanical (filters and barriers), and chemical (chemical exposure) (Van Eenennaam and Wong, 2008).

The most common methods of mechanical containment are implemented in the culture of finfish species, and include the use of sea cages and net pens (Van Eenennaam and Wong, 2007). Net pens and sea cages are however susceptible to bad weather conditions, presence of predators, wear and tear, industrial accidents, and are therefore not highly effective as proper containment methods (Fredriksson *et al.,* 2008; Naylor *et al.,* 2005; Wong and Van Eenennaam, 2008).

Closed containment methods such as recirculating systems and quarantine include a variety of production systems with varying degrees of isolation to eliminate the contact between farmed fish and the surrounding aquatic environment (Fredriksson *et al.,* 2008). These closed containment methods however require more energy to run than standard methods (Boulet *et al.,* 2010).

Quarantine, a method of closed mechanical containment, also has a risk of releasing toxic substances, used in treatment protocols, into the environment if the management and methodology are not properly implemented and managed (Van Eenennaam and Wong, 2008).

Another proposed closed containment method is geographic isolation, where closed land-based systems housing transgenic or polyploid fish are completely closed off from the surrounding environment. Unfortunately geographic isolation methods are often economically non-viable due to high operational costs (Maclean *et al.,* 2002).

Chapter 3 reported on the use of triploidy as a form of biological containment through induced sterility (Allen and Downing, 1986; Cal *et al.,* 2006; Piferrer *et al.,* 2009). The results, however, indicated that the induction technique was not effective in inducing true sterility in HM, although arrested gonadal development was observed. The presence of mature gametes observed in triploid HM gonads in the above mentioned study, creates a concern relating to the possible hybridization that might occur should GM abalone escape from

commercial flow-through systems. The potential of mechanical and chemical containment methods to contain GM abalone need to be investigated.

In South Africa, the culture of HM takes place in land-based open continuous flow-through systems, which requires large volumes of water. Water is pumped from the ocean, where it passes through a sand- and/or micro-filter system before it is distributed to the hatchery and grow-out sections of the farm (Paulet, 2011). The current design and function of open continuous flow-through systems in commercial HM production presents various potential routes along which the life stages housed in the system can escape, and be introduced back into the ocean. Water drainage from the respective sections all collectively drain into a main farm drainage system, which flows directly back into the ocean (Figure 4.1).

The four HM life stages that are housed within commercial systems include male and female gametes, larvae, settlement larvae and spat, all of which differ in behaviour and size. The differences in the size and behaviour of the life stages necessitates the development of tailor-made mechanical containment methods for each life stage.

Little information is available on mechanical containment methods, as well as the implementation of methods into existing open continuous flow-through abalone production culture systems.

The aims of this study was therefore to determine the efficacy of 100 µm polypropylene filter bags to contain HM gametes, larvae and settlement animals in an existing commercial production system, and to assess the efficacy of an obstructive material to impede the escape of weaning animals (spat) from their holding tanks during increased nocturnal activity.

## 4.2 Materials and methods

### 4.2.1 Experimental location

The study was conducted at the HIK abalone farm, Hermanus, South Africa (34°26'1.60"S and 19°13'14.06"E). The farm incorporates all stages of commercial production, including a hatchery, weaning, and grow-out section. Due to the Farm's (HIK) proprietary information, stocking densities, certain time periods and flow rates of housing sections cannot be disclosed.

### 4.2.2 Experimental animals and housing facilities

The hatchery is divided into four sections, i.e. a broodstock area, a larvae area, a settlement area, and the weaning area, where each of the respective life stages are housed and managed separately (Figure 4.1).

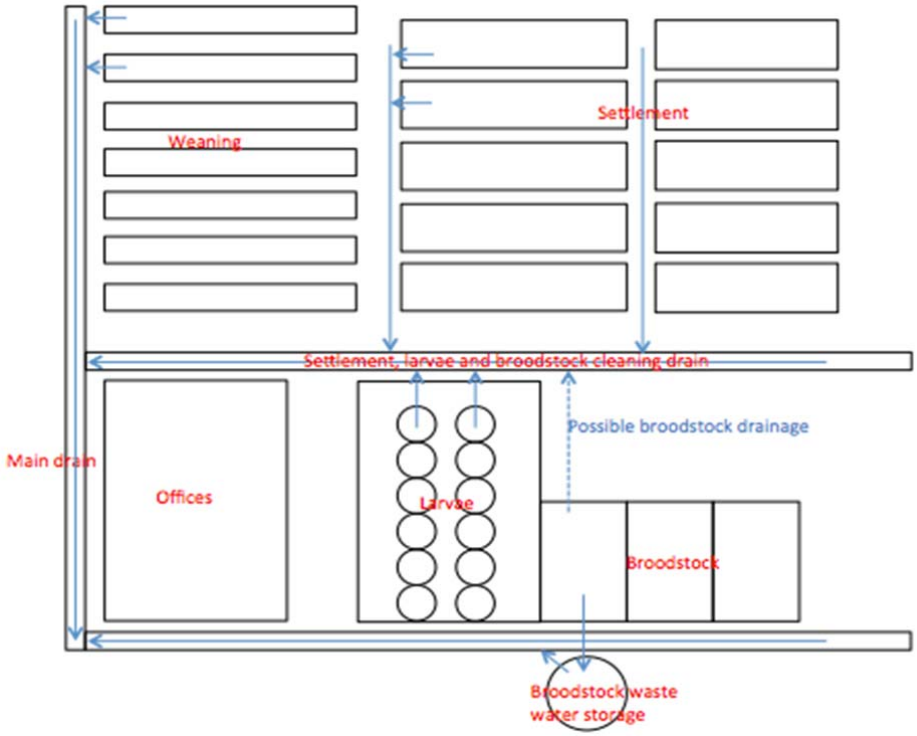


**Figure 4.1.** A diagram depicting the different sections of the HIK hatchery as well as the flow pattern of water as it exits each housing section to flow to the ocean (Paulet, 2011).

Water is distributed to the hatchery and grow-out sections of the farm from a header tank (Paulet, 2011). Seawater is pumped in from the ocean and filtered through a sand- and micro-filter system in the header tank before distribution to the various sections. Depending on the water specifications for the specific life stage, the water can be re-filtered. Water is circulated through the tanks of the life stages housing systems at specific flow rates and then drains into a central draining system, via PVC pipes leading into concrete channels and pipe systems, directing the effluent water back to the ocean as seen in Figure 4.1.

Current on-farm containment methods include a 120 µm banjoe sieve, which consists of an overflow pipe covered with 120 µm mesh, positioned inside the outlet pipe of each larval rearing tank and a 0.3 cm mesh placed over the standpipe of the weaning tanks, as these animals have a tendency to crawl out of the tanks at night. No additional or alternative methods of containment are currently in place in the broodstock and settlement sections.

Due to the limited availability of animals and time constraints, treatments could not be repeated; therefore additional samples and measurements were taken at each treatment to decrease the variation.

*4.2.2.1 Broodstock housing section*

Male and female broodstock animals are housed separately in plastic tanks at varying stocking densities. Farm protocol houses female abalone (HM) in tanks at densities of one, three or five animals per tank, and male animals are maintained at a stocking density of five animals per tank.

Broodstock are induced to spawn at six-week intervals, according to the sodium hydroxide method described by Visser-Roux (2011). Males begin spawning approximately four hours after the induction and females approximately six hours after induction. Ova, which range between 260 - 350 µm (Visser-Roux, 2011), are negatively buoyant and settle on the bottom of the tank. Sperm, which are 2.5 - 3.5 µm in length, and 0.5 µm in diameter (Visser-Roux, 2011), swim evenly throughout the male tanks after being released. The production protocol is to allow fertilization to occur within the female tanks, after an arbitrary volume of sperm is added to the female tanks (Paulet, 2011). For the purpose of this study unfertilized ova were used to assess the efficacy of filter bags to contain ova.

Water flows into the tanks at a specific flow rate through inlet pipes. A standpipe fits into the middle of each tank, which empties the effluent water (containing gametes) into a collective gutter which runs along the front of the broodstock tanks, and joins to a central down pipe. The central down pipe flows into the cement drain, and out of the broodstock room (see Figure 4.2) linking to the main farm drain and flowing directly into the ocean. Currently there are no containment barriers within the water drainage system of the broodstock room, creating a high risk of gametes escaping directly into the environment.

*4.2.2.2 Larvae housing section*

Fertilised ova are transferred from the broodstock tanks and stocked at specific larval densities in inverted cone-shaped fiberglass hatch-out tanks, arranged closely in rows. Each tank has an outlet pipe situated above a cement drain (See Figure 4.3). Larvae are housed here for approximately five days until they undergo metamorphosis from veliger larvae into trochophore larvae, which will settle soon after metamorphosis (Grubert, 2005). Larvae swim around near the surface of the water level, as larval rearing tanks are open at the top. Veliger larvae range from 300 - 350 µm in length and trochophore larvae are 300 µm in length (BHCAP OceanLink, 2012).

Water flows into the tanks through pipes positioned above the larvae tanks at specific flow rates. Effluent water from the tanks flows directly out the outlet pipe into the cement drain and out of the larvae room, linking to the main farm drain and flowing directly into the ocean. Larval rearing tanks include an 120 µm banjoe sieve (an overflow pipe covered with mesh) placed within the down pipe of the larvae tanks as a practical method of securing larvae within the production system.

Larvae are therefore not considered a high escape risk, but because larvae undergo metamorphosis and settle, they are considered to have the highest possibility of settling in the wild environment if they were to escape in the effluent water.

*4.2.2.3 Settlement housing section*

Six days post hatch larvae are transferred and stocked at specific densities in plastic rectangular settlement tanks. Settlement tanks, which are arranged in rows in the housing area, contain racks of plastic corrugated sheets coated in diatoms. Once larvae have been added to the settlement tanks, the inflow water is turned off for a certain period of time, before the water is turned on again. Once the water is turned on, larvae remain in the settlement tanks for a further period of time. During this time the larvae undergo a metamorphic phase, which is a gradual process described by a change in anatomy and physiology of the animal (Medina, 2009), and this can take about seven days to complete (Leighton, 1974). Once metamorphosis is complete, animals attach themselves to the diatom plates and begin feeding on the diatoms. Settlement larvae range between 350 - 700 µm in diameter (BHCAP OceanLink, 2012).

Water flows into the tanks through pipes positioned above the settlement tanks at specific flow rates. Each tank contains a standpipe positioned above a cement drain, the effluent water that runs over the standpipe (Figure 4.4B) flows directly into the drain and out of the hatchery, linking to the main farm effluent system and flowing directly into the ocean. There are no current containment barriers within the flow pattern of the settlement area, creating an opportunity for the larvae to escape directly into the environment.

*4.2.2.4 Weaning housing section*

Weaning animals (spat) are transferred from the settlement section and stocked at specific densities in shallow square plastic containers placed approximately 1 m above the ground on wooden tables. Each tank includes a standpipe placed directly over a cement drain. Tanks contain black plastic cones, which spat use to hide away from daylight. Spat range between 700 – 1 000 µm in size (BHCAP OceanLink, 2012).

Water flows into the tanks through pipes positioned above the weaning tanks at specific flow rates. The effluent water from the tanks flows down the standpipe, into the cement drain and out of the weaning area, linking to the main effluent system (Figure 4.1) and flowing directly to the ocean. Because spat are larger than the other life stages, the occurrence of them escaping in effluent water is less of a concern. The concern with spat is the escape (crawling) over the sides of their tanks and down the standpipes. They are known to crawl into the drains leading to the ocean. Current containment barriers included in the weaning tanks are 0.3 cm plastic mesh placed over the standpipes.

### 4.2.3 Experimental protocols and design

*4.2.3.1 Containment of ova by means of polypropylene filter bags*

The experimental design accommodated the different densities at which females are stocked. Female broodstock are housed in tanks at densities of one, three or five animals per tank. The broodstock available for the trial were housed at a density of one female per tank, therefore the number of gametes that one, three or five female animals would collectively spawn was mimicked by simultaneously emptying one, three or five singularly stocked tanks.

The potential of a polypropylene filter bag (Filcon, South Africa) with a pore size of 100 µm to contain ova was evaluated for the three densities. Effluent water was redirected from the central gutter into 25 L buckets using a modified pipe attachment that fitted onto the end point of the central gutter (Figure 4.2). A filter bag was placed inside a 25 L bucket, and fitted securely to the handle of the bucket with a cable tie. The effluent water was drained through the 100 µm filter bag and collected into a 25 L bucket.

Once all the effluent water had passed through the filter bag into the collection bucket, the collected ova were allowed to settle along the bottom of the bucket. A serological pipette (Lasec, South Africa) was used to draw ova from the bottom of the bucket, and the content was transferred into pre-labelled 15 mL Falcon tubes (Lasec, South Africa). Seven samples from each treatment were collected for analysis. The contents of each Falcon tube was transferred to individual Petri dishes (Lasec, South Africa), and analysed for the presence/absence of ova by using a light microscope (Olympus CX21). For each sample the number of ova was recorded.


**Figure 4.2.** A: The modified pipe collecting effluent water from male and female broodstock tanks. B: A filter bag filled with ova.

The containment of sperm by means of the polypropylene bags were not evaluated in this study, as the smallest available pore size was 0.5 µm, the same as the diameter of the sperm head.

*4.2.3.2 Containment of larvae by means of polypropylene filter bags*

The experimental design tested the containment of four larval densities, namely a low larval density (1 larvae/mL), a medium larval density (3 larvae/mL), a high larval density (6 larvae/mL), and a control (2 to 3 larvae/mL), which is a standard stocking density used on the farm. The 100 µm polypropylene filter bags (Filcon, South Africa) were of a smaller pore size than the current on-farm containment; a 120 µm banjoe sieve placed in the outlet pipes of larvae tanks, to ensure that no larvae could potentially escape.

The banjoe sieves were only removed from the outlet pipes of larval tanks for the duration of the experiments and remained in the control treatment larval tank. The 100 µm filter bags were placed in three separate 25 L buckets and secured to the downpipes with cable ties. A filter bag was not placed in the collection bucket of the control treatment. Buckets were positioned below the outlet pipes of the larval tanks. To ensure that no

larvae would swim over the top of the filter bag and into the effluent water bucket, the rim of the filter bags were raised above the water level (Figure 4.3), to ensure no larvae could escape through the top opening of the filter bags.

Effluent water samples were collected 5 cm below the water level of the 25 L bucket as larvae are inclined to swim towards the surface of a container. Seven water samples from each treatment bucket were collected in pre-labelled 15 mL Falcon tubes (Lasec, South Africa) each morning at a fixed time, for five days. The contents of each Falcon tube was transferred to individual Petri dishes (Lasec, South Africa), and analysed for the presence/absence of larvae by using a light microscope (Olympus CX21). For each sample the number of larvae was recorded.

**Figure 4.3.** Filter bags placed underneath cone-shaped larvae tanks.

*4.2.3.3 Containment of settlement larvae by means of polypropylene filter bags*

The experimental design included two treatments, the efficacy of a polypropylene filter bag (Filcon, South Africa) with a pore size of 100 µm to contain settlement larvae and a control treatment (a settlement tank with no filter bag) over a period of seven days. The standpipe of the settlement tank was modified to accommodate a 100 µm filter bag on the inside (See Figure 4.4A) of the pipe. The filter bag was secured with cable ties to ensure effluent water would only be directed through the filter bag (Figure 4.4A). The diameter of the standpipe was not considered to affect escape behaviour of the larvae, as the same volume of water would flow over the modified standpipe as the standard standpipe found within a settlement tank. A 5 L bucket was positioned below the outlet pipe of the settlement tank to collect the effluent water; for the space below the settlement tanks was too small to accommodate a 25 L bucket.

Effluent water samples were collected 5 cm below the water level, around the edge of the 5 L bucket as larvae are inclined to swim towards the sides of the container as they are looking to settle. Seven water samples from each treatment bucket were collected in pre-labelled 15 mL Falcon tubes (Lasec, South Africa)

each morning, for seven days. The contents of each Falcon tube was transferred to individual Petri dishes (Lasec, South Africa), and analysed for the presence/absence of larvae by using a light microscope (Olympus CX21). For each sample the number of larvae was recorded.

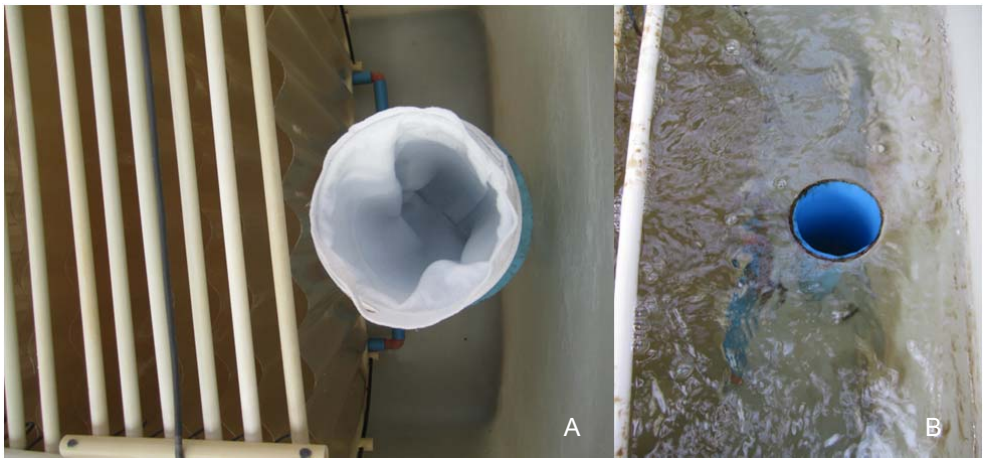


**Figure 4.4.** A: modified standpipe in the settlement tank. B: A normal standpipe in the settlement tank.

*4.2.3.4 Containment of spat by means of obstructive materials*

The experimental design included three treatments, the efficacy of three obstructive materials namely, Astroturf (Top Carpets & Floors, Hermanus, South Africa), carpet (Top Carpets & Floors, Hermanus, South Africa) and Velcro (Hermanus Factory shop, Hermanus, South Africa) and a control. Each experiment was repeated three times. The use of obstructive materials to contain spat in the weaning tanks was chosen on practical considerations and recommendations by Paulet (2011), as spat are known to crawl down the standpipes and over the edge of the weaning tanks and escape through the cement drains. The current on-farm method of containment includes a 0.3 cm mesh placed around the standpipe of each weaning tank.

The plastic rectangular weaning tanks, which house the spat, have a 5 cm lip around the outside circumference (See Figure 4.5). Obstructive materials were affixed to the entire circumference of the 5 cm lip using a marine sealant (Bostik), ensuring no openings. The 0.3 cm mesh placed around the standpipe of the weaning tanks remained in place for all treatments, including the control. Once materials were secured, tanks were refilled with filtered seawater. A total of 100 spat were added to each treatment tank and observed for 48 hours. After the 48 hours, animals from each treatment tank were anesthetized using magnesium sulphate (Paulet, 2011) and re-counted to determine the number of escapees.

**Figure 4.5.** A standard rectangular weaning tank shown with carpet offcuts glued to the edges of the tank, using marine sealant, to obstruct spat from escaping.

### 4.2.4 Statistical analysis

As there were unbalanced number of observations per group, the Least Square Means (LSM) were calculated to interpret the results. The data for the ova treatments were analysed using Analysis of Variance of SAS for Windows version 9.3 (2011). Due to the unbalanced number of observations Type III sum of squares were calculated using PROC GLM in order to analyse the number of escaped animals per life stage and to obtain the Least Square Means (LSM) and the Standard Error of the Means (SEM).

## 4.3 Results

### 4.3.1 Containment of ova by means of polypropylene filter bags

A statistically significant difference ($P < 0.05$) was noted between the number of larvae observed in the effluent water samples of the three treatment groups (Table 4.1). A stocking density of one female per tank resulted in the least number of escapees 16.9 ± 32.9 ($P > 0.05$) when compared to the other stocking densities (Table 4.1).

**Table 4.1.** The comparative LS means (LSM) and standard error of the mean (SEM) for the number of escaped ova from three treatment groups (one, three and five animals) which passed through a 100 µm polypropylene filter bag.

| Treatment | | LSMean Ova | SEM |
|---|---|---|---|
| **Number of animals per tank** | 1 | 16.9[a] | 32.9 |
| | 3 | 36.3[a] | |
| | 5 | 537.0[b] | |

[a,b,c] Rows with different superscripts differ significantly (P>0.05)

The collective ova from a density of three female abalone indicated an LSM of 36.3 ± 32.9 escapees observed per effluent treatment sample, which did not differ significantly from the density of one female animal per tank ($P > 0.05$). Ova spawned by five female animals per tank resulted in a significantly higher number of observed escapees with an LSMean of 537.0 ± 32.9 ($P < 0.05$) escapees observed per effluent treatment sample.

### 4.3.2 Containment of larvae by means of polypropylene filter bags

The filter bags and the banjo sieve method did not differ ($P > 0.05$) in terms of the method's ability to contain the abalone larvae in this study. The 100 µm polypropylene filter bags were effective in containing the larvae at all three densities. No larvae were observed in the samples collected from the low larval density (1 larvae/mL), medium larval density (3 larvae/mL) and high larval density (6 larvae/mL). No larvae were observed in the control treatment (banjo sieve).

### 4.3.3 Containment of settlement larvae by means of polypropylene filter bags

The larvae data obtained was analysed per day, as the co-efficient of variation (CV = 95.1) was large, and data was not normally distributed. A statistical significant difference in the number of settlement larvae was only noted between the control (no filter bag) and the treatment (100 µm polypropylene filter bag) on day one of the trial ($P = 0.002$), with an average number of 3.71 ± 0.06 larvae observed per effluent water sample. No significant difference ($P = 0.20$) was observed between the number of larvae observed in the control (no filter bag) and the treatment (100 µm polypropylene filter bag) on day two (0.57 ± 0.06) of the trial, and further no larvae were observed from day three (0) to day seven (0).

### 4.3.4 Containment of spat by means of obstructive materials

There were no statistically significant differences ($P = 0.69$) observed in the number of animals retained in the weaning tanks after the 48-hours, between the control (no obstructive barrier) and the three obstructive materials (AstroTurf, carpet and Velcro).

**Table 4.2.** The comparative LSMeans (LSM) and standard error of the mean (SEM) for the number of spat retained after a 48-hour period when three obstructive materials were sealed to their tanks.

| Treatment | Animals retained | SEM |
|---|---|---|
| AstroTurf | 99[a] | 0.67 |
| Carpet | 100[a] | |
| Velcro | 99[a] | |
| Control | 99[a] | |

[a,b,c] rows with different superscripts differ significantly ($P>0.05$)

The use of a carpet barrier retained all treatment animals (100 ± 0.67), and AstroTurf, Velcro, and the control (no obstructive barrier) indicated an insignificantly lower number of retained animals (99 ± 0.67; Table 4.2).

## 4.4 Discussion

This study investigated the efficiency of 100 µm polypropylene filter bags, and obstructive materials (AstroTurf, carpet and Velcro) as potential methods to mechanically contain the life stages of *Haliotis midae* (HM) housed within a hatchery, from escaping a flow-through production system. Assessment of different methods of containment was deemed necessary on the basis that GMOs escaping from production systems could cause potential harm on the surrounding natural environment.

During the study, the gamete stages were considered the most critical point for escapees, as no current containment methods are in place in the broodstock rooms. Followed by larvae, which have the possibility of settling in the natural environment if they escaped, as well as spat which have a tendency to crawl out of their weaning area, and move towards the ocean via the cement drains. Spat are considered critical as they could potentially grow to sexual maturity, and disrupt the genetic diversity in the natural environment.

Little information is available on mechanical containment methods in the aquaculture industry, and abalone in particular. The use of filter bags was chosen as the mechanical method to contain HM in an open continuous flow-through hatchery system, as filter bags did not require excessive modification of the existing production system on the farm where the study was conducted.

In this study the 100 µm polypropylene filter bag showed the highest efficacy of containment when gametes from one broodstock female abalone was passed through the filter bag (16.9 ± 32.9 average ova observed), with a decreased efficacy when gametes at higher densities from sets of three and five female abalone respectively passed through the filter bags (36.3 ± 32.9 and 537.0 ± 32.9 average ova observed per sample).

The 100 µm polypropylene filter bags proved to be ineffective in providing full containment of ova produced by broodstock housed at densities of three females and higher. The use of 100 µm polypropylene filter bags can therefore not be considered as a secure method for the containment of female broodstock gametes in abalone hatcheries.

Female abalone, depending on their age, will release between 1 and 10 million ova per spawning cycle (FAO, 1990). When this number is compared to the average number of escaped ova observed per treatment when ova from one female abalone was contained using a 100 µm polypropylene filter bag (16.9 ± 32.9), it is incomparable to the vast number of ova that the filter bag was able to contain. However, the purpose of mechanical containment is required to contain 100% of ova as a precautionary approach, given the yet unknown success of escaped gametes to contribute to successful fertilisation and settlement in the natural environment. The use of a containment method within the broodstock area must be a permanent fixture to ensure containment during sporadic spawnings, which can be triggered by a number of factors, such as an increase in seawater temperature. Therefore the use of filter bags within the broodstock room is considered an impractical approach to mechanical containment, as filter bags are also prone to blockages (Hawkins and Jones, 2002), and requires cleaning or replacement after a single use.

The 100 µm polypropylene filter bags did not produce superior results in the containment of larvae, when compared to the use of a 120 µm banjoe sieves. No larvae were observed in the effluent water samples collected from the treatment groups, as well as the control treatment (Banjoe sieve).

The settlement treatment indicated that 100 µm polypropylene filter bags proved to be effective in containing the settlement larvae on the first day when the water in settlement tanks are turned on. The larvae observed on day one (3.71 ± 0.06) in the control treatment (no filter bag) was expected, as it relates to a production practice where the settlement tanks are a closed system for a certain period of time before the water is turned on, therefore larvae which do not settle within this time period are flushed out of the system on the first day.

The reason for larvae not settling on diatom plates is unknown; settlement rates in abalone culture are said to range between 30 - 60% (Duame, 2003), which means the other 40 - 70% are being flushed out into the ocean. Therefore precautionary containment methods should be further investigated for implementation on the first two days of the settlement schedule. Hawkins and Jones (2002) and Theil *et al.* (2004) hypothesized that the probability that escaped larvae settling in the wild is considered low, although this has not been fully investigated (Theil *et al.,* 2004).

Obstructive materials used to contain spat showed no significant differences to the control treatment (no obstructive materials); however, animals were observed below the lip of the tanks where the obstructive materials were affixed. The potential of obstructive materials to contain spat needs to be evaluated over a longer period of time, as 48 hours may not have been long enough to assess their behaviour. The behaviour and movement of spat is dependent on the seawater quality and temperature (Sales and Britz, 2001; Steinarsson and Imsland, 2003; Paulet, 2011). It is observed that when the seawater temperature declines to below 18ºC, the activity of spat increases, and a higher number of spat escapees are observed in the surrounding drains and on the floor of the hatchery (Paulet, 2011).

Other possible methods of mechanical containment methods, which have been proposed, for the aquaculture industry includes micro screens, filter bags, cartridge filters and sand filters, which are categorized by filtration performance, and flow rates (Colt and Huguenin, 2002). A sand filter, hypothetically, would be an appropriate method for incorporation in an abalone hatchery, as sand filters can filter a wide range of particle sizes. The use of a coarse sand filter will likely be able to filter larvae and ova, and clay will allow for finer filtration (Colt and Huguenin, 2002), which would be ideal for the containment of sperm, which could not be assessed in this study. Although the use of a sand filter will potentially contain each life stage of abalone according to their size, sand filters requires intensive labour, and high operating costs (Colt and Huguenin 2002; Wong and Van Eenennaam, 2008). Other considerations for implementing sand filters include the rate of clogging, which is dependent on the volume of water, and the suspended particles flowing through the sand filters, which could decrease its efficacy and be ineffective (Colt and Huguenin, 2002).

These authors also stated that incorporating a sand filter into a system, which it has not been designed for, could cause incompatibilities within the system, such as space constraints.

Absolute containment, the requirement for an effective mechanical containment method, was only observed in the settlement larvae and one-day old larvae treatments. Although 100 µm polypropylene filter bags were effective in containing larvae, additional containment in the larvae section is unnecessary, as the current on-farm containment method using banjoe sieves is adequate in this regard.

The use of a filter bag could be considered as a containment method in the settlement area, as a single filter bag can be used for the first two days of settlement, and this should bear no cost implications as only a few number of tanks are settled per production schedule; filter bags can therefore be cleaned and reused during the next settling period.

An alternative method of containment is required for the broodstock section, i.e. the possible use of a sand filter. Depending on the spatial requirements, future investigation on the use of a sand filter at major points of drainage links may be effective and therefore containment at each life stage housing section may not be necessary.

## 4.5 Conclusion

The objective of this study was to determine the efficacy of 100 µm polypropylene filter bags and obstructive materials (AstroTurf, carpet and Velcro), as potential mechanical methods to contain the four life stages of *Haliotis midae*, namely; ova, larvae, settlement larvae and spat, housed in an existing open continuous flow-through hatchery production system.

The use of 100 µm polypropylene filter bags could not fully contain female broodstock gametes, as ova were observed in the effluent water samples. The number of ova observed in effluent water samples increased as the female densities of the treatments increased. The polypropylene filter bags, were effective in containing one-day-old larvae as well as settlement larvae, as no larvae were observed in the sampled effluent water of the one-day-old larvae and settlement larvae treatments.

The use of obstructive materials to contain life stages beyond settlement showed no significant ability to contain spat over the 48-hour treatment period.

The inability of polypropylene filter bags to contain ova indicates the need for further investigation into other methods of mechanical containment (i.e. sand filters) for absolute containment of female broodstock, as this stage is considered one of the more critical stages that need attention.
The use of obstructive materials must be further tested over a longer period of time. Investigation into the use of a sand filter, not only for the broodstock room, but as a possibility to implement into an existing open

continuous flow-through abalone production system at points of major linkage in the drainage system should be investigated. This could decrease the implementation costs as well as decrease the labour intensity of containment methods at each housing area.

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# Chapter 5

# Chemical containment of abalone (*Haliotis midae*)


## Abstract

Commercial production of abalone, (*Haliotis midae*), is characterized by an interval of up to five years to from hatchery to harvest. The slow growth rate is a result of the animals partitioning energy towards gonad development once the animals enter puberty. The production of triploid abalone provides an opportunity to improve the cost-efficiency of production by shortening the time to harvest, as triploid animals are believed to divert their energy into somatic growth rather than gonad development. The production of genetically modified abalone, however, poses a risk of organisms escaping into the natural environment. The implementation of containment methods in abalone culture systems can potentially assist in the control of the escape of farmed animals, and prevent and/or minimize the effect of genetically modified abalone on wild stocks, and the natural environment. In South Africa, commercial abalone operations use open continuous flow-through systems, and the different life stages (i.e. spermatozoa, ova, larvae, spat, immature and mature abalone) would require specific containment strategies to ensure effective containment of genetically modified abalone in such systems. No chemical containment methods have yet been developed for the containment of abalone. This study therefore investigated the efficacy of chlorine-based chemicals, i.e. sodium hypochlorite and Biox™, to potentially contain all life stages present in abalone hatcheries. Abalone life stages were exposed to the two chemicals at varying concentrations (10 ppm, 50 ppm and 100 ppm), over five exposure times (4, 6, 8, 10 and 12 minutes). Sodium hypochlorite and Biox™ affected the viability and survivability of the five abalone life stages maintained in an abalone hatchery. Although no significant differences were observed in the efficacy of the two chemicals, higher concentrations of each chemical resulted in higher mortality rates in all life stages of the abalone. The appropriate method of chemical administration under field conditions should be further evaluated.


## 5.1 Introduction

Research efforts in the national abalone industry focus on the use of biotechnologies such as gene transfection, and the development of techniques that can contribute to shortening the time it takes HM to reach a market size of 70 to 100 g. Due to many factors, on average it takes HM individuals three to five years to reach this size (Hahn, 1989).

The induction of triploidy, a genetic modification technique that results in HM retaining a third set of chromosomes, presents a possible advantage to shorten the interval to harvest. Triploidy is associated with sterility, and a faster growth rate due to metabolic energy being diverted from gonadal development to somatic growth (Allen and Downing, 1986; Beaumont and Fairbrother, 1991; De Beer, 2004; Liu *et al.,* 2004). Another advantage attributed to triploidy is an improved meat quality and taste, through the prevention of glycogen depletion during the spawning season (Allen and Downing, 1986; Beaumont and Fairbrother, 1991; Boudry *et al.,* 1998; De Beer, 2004; Liu *et al.*, 2004; Morrissey and Rasmussen, 2007; Xie *et al.,* 2008).

The potential introduction of triploid HM from farms into the surrounding wild populations is a cause for concern with regard to the commercial application of triploidy in commercial abalone culture systems. The interactions between wild stocks and triploid animals include risks associated with the modification of the genetic diversity of the wild stocks, as well as transfer of superior traits that are associated with modified organisms. Traits that can give genetically modified organisms (GMOs) an advantage include an increased reproductive ability, increased growth rate, increased disease resistance, and increased predation ability (Muir and Howard, 1999; Maclean and Laight, 2000; Hawkins and Jones, 2002). The use of effective containment methods can minimize or prevent the effect of escaped genotypes from a production system (Morrissey and Rasmussen, 2007) to the surrounding wild population. Transgenic animals can be contained by one or a combination of three methods of containment, i.e. biological techniques resulting in incomplete sterility, mechanical methods that can include e.g. filters and barriers, and chemical methods which entails exposure of the animals to chemicals that usually have a lethal or toxic effect (Van Eenennaam and Wong, 2008).

In Chapter 3 it was concluded that triploid induction by the hydrostatic pressure method was not effective in inducing true sterility, although arrested gonadal development and function was observed. As mature ova and sperm were observed in the gonads of 75-month-old triploid HM in the abovementioned study, it raises concerns over the possible impact of escapees on the surrounding environment and wild stocks relating to the possible hybridization that might occur should genetically modified (GM) abalone escape from commercial flow-through systems.

Currently, production animals, which are genetically diploid, are also exposed to genetic selection in an effort to enhance the efficiency of production through specific traits, such as settlement ability and growth rates. This type of selection can similarly cause a genetic shift in wild populations if interactions occur (Devlin e*t al.*, 2006). This indicates a need for a containment strategy within the abalone industry regardless of the genotype of farmed HM, whether triploid or genetically selected diploids.

The principle of chemical containment is based on the exposure of animals to chemicals for a specific contact time, with the chemicals designed to have toxic or lethal effects. Chemicals are designed to cause a 100% mortality rate in specific life stages upon application (Wong and Van Eenennaam, 2008). Aquatic organisms may have narrow tolerance ranges for survival within their environment, and therefore a limited ability to offer resistance to the effect of chemicals (Wong and Van Eenennaam, 2008). This trait can potentially be exploited in establishing the potential of certain chemical agents to be used as a form of containment. The other concern is that the application of such chemicals in an open flow-through system can cause adverse effects on the ecosystem in addition to the effect on the species (Chen and Liao, 2004). The European Union Risk Assessment Report (2007) indicated that chlorine dioxide and sodium hypochlorite are two chemicals that cause little harm to the environment (Simpson *et al.,* 1993). Sodium hypochlorite is highly reactive and unstable, and will rapidly disappear in the aquatic environment (European Union Risk Assessment Report, 2007).

Chlorine dioxide, also known as Biox™ (Immunovet, South Africa), has been used in a number of industries, including the dairy industry, beverage industry, the pulp and paper industry, the fruit and vegetable industry, various canning plants, the poultry industry, and the beef processing industry as disinfectants. Chlorine dioxide is also used in municipal potable water treatment facilities (Simpson *et al.,* 1993).

Little information is available on the potential of chemical containment methods to limit, and ultimately prevent, the potential negative effects that GM genotypes may have on the surrounding wild populations. The aim of this study was therefore to investigate the potential of Biox™ and sodium hypochlorite to be used to chemically contain all respective life stages of HM, i.e. ova, sperm, larvae, and spat in commercial culture systems.

## 5.2 Materials and methods

### 5.2.1 Experimental location and facilities

The study was conducted at the HIK Abalone Farm, Hermanus, South Africa, (34°26'1.60"S and 19°13'14.06"E). Due to the Farm's (HIK) proprietary information, stocking densities, certain time periods and flow rates of housing sections cannot be disclosed.

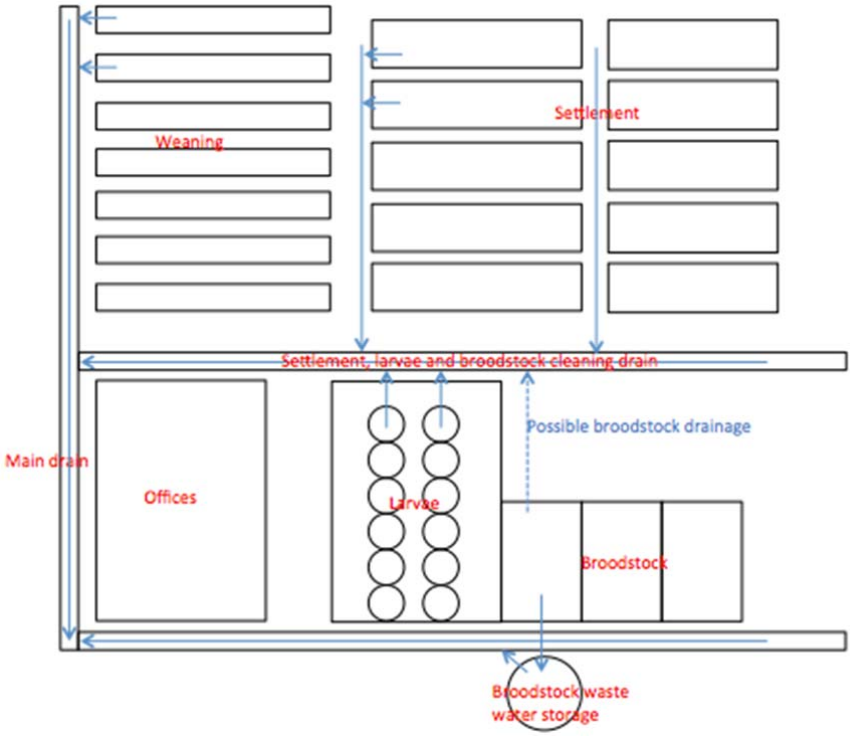


**Figure 5.1**. A diagram depicting the different sections of the HIK hatchery as well as the flow pattern of water as it exits each housing section to flow to the ocean (Paulet, 2011).

The HIK abalone hatchery is divided in four main sections associated with each of the respective life stages, namely a broodstock section, a larval section, a settlement section, and a weaning section. The layout of the continuous flow-through hatchery system is presented in Figure 5.1.

Seawater is pumped into a header tank from the ocean, where water is filtered to 90 micron. From the header tank, water is distributed to the specific sections on the farm where additional treatment (filtration, aeration, heating, etc.) may be conducted depending on the specific requirements of the particular life stage. Water is drained continuously from each housing unit and section into a main drainage system, which channels the effluent water back to the ocean.

*5.2.1.1 Broodstock housing section*

Male and female broodstock animals are housed separately in plastic tanks at varying stocking densities in the broodstock section. Females are maintained in tanks at three densities (one, three and five animals per tank), and male animals are maintained at a stocking density of five animals per tank.

Water is pumped at specific flow rates into the male and female broodstock tanks. Each tank has an inlet pipe and a standpipe, fitted in the middle of each tank. Broodstock tanks empty effluent water (containing gametes) into a collective gutter, which runs along the front of the tanks and joins a central down pipe. The central down pipe flows into a cement drain, and water flows out the broodstock room linking to the main farm drainage system, which flows directly into the ocean.

There are no current containment barriers within the water drainage system of the broodstock section, creating a high risk of gametes escaping directly into the natural environment. Ova, which range between 260 – 350 µm in size (Visser-Roux, 2011), are negatively buoyant, and settle on the bottom of their tanks. Sperm, which are 2.5 - 3.5 µm in length, and 0.5 µm in diameter (Visser-Roux, 2011), swim evenly throughout the male tanks after spawning.

*5.2.1.2 Larvae housing section*

Larvae are housed in inverted cone-shaped fibreglass tanks in a separate room. The inlet pipes of the larvae tanks are positioned on top of the larvae tanks where filtered seawater flows in at a specific flow rate. Each tank has an outlet pipe positioned above a cement drain. The effluent water from each tank flows down the outlet pipe and into the cement drain. The effluent water then flows out of the larvae room, linking to the main farm drain and flowing directly into the ocean.

Currently, a 120 µm banjoe sieve (an overflow pipe covered with mesh), is fitted into the down pipe of the larval rearing tanks, as a means to contain the larvae within the production system. Larvae are therefore not considered a high escape risk, but due to the behaviour of larvae, they are considered to have the most possibility of settling in the wild environment if they were to escape in the effluent water. Larvae metamorphose and settle on a substratum. During the larval rearing stage, larvae swim evenly near the

surface of the tanks. Veliger larvae range between 300-350 µm in length, and trochophore larvae are on average 300 µm in length (BHCAP OceanLink, 2012).

*5.2.1.3 Settlement housing section*

Settlement tanks are arranged in rows in the settlement section, and contain plastic corrugated sheets covered with diatoms, which serve as settlement substrate for the larvae. Each tank has an individual inlet pipe where filtered seawater flows in at a specific flow rate. Effluent water flow is over the standpipe, and down into the cement drain. The cement drains links to the main farm drain and water is directed towards the ocean.

There are no current containment barriers within the flow pattern of the settlement area creating an opportunity for the larvae to escape directly into the environment. Settlement larvae range between 350 - 700 µm in diameter (BHCAP OceanLink, 2012).

*5.2.1.4 Weaning housing section*

Spat (weaning animals) are housed in shallow square plastic containers placed approximately 1 m above the ground on wooden tables, and are stocked at specific densities. Each tank has a standpipe placed directly over a cement drain. Filtered seawater flows into the tanks through inlet pipes positioned above the weaning tanks at a specific flow rate. The effluent water from the tanks flows down the outlet standpipes, and into the cement drain below. The cement drain links to the farms main drainage system, and channels the effluent seawater directly to the ocean. Spat range between 700 - 1000 µm (BHCAP, OceanLink, 2012) in size. The occurrence of them escaping in effluent water is unlikely, but they are known to escape over the sides of the tanks and down the standpipe.

Currently spat are contained in the weaning tanks using 0.3 cm plastic mesh placed over the standpipes, as spat are known to crawl down the standpipe into the drains at night.

### 5.2.2 Experimental animals

*5.2.2.1 Artificial spawning of broodstock and collection of gametes* (*Haliotis midae*)

Broodstock of male and females, with an average length of 20 ± 5.0 cm and weight 1.5 ± 0.5 kg were used in this study. Broodstock were induced to spawn according to the sodium hydroxide method described by Visser-Roux (2011). Sodium hydroxide is added to the tanks to increase the pH of the water, with hydrogen peroxide being added 15 minutes after the addition of the sodium hydroxide (Visser-Roux, 2011). Three hours later, residual hydrogen peroxide is removed by flushing the system to avoid poisoning of the animals (Morse *et al.,* 1977). Tanks are then filled with clean filtered seawater, and the water aerated according to the specifications of the broodstock husbandry protocol (Visser-Roux, 2011). Males begin to spawn approximately four hours after being induced, and females approximately four hours after the males. The production protocol is to allow fertilization to occur within the female tanks, after an arbitrary volume of sperm

is added to the female tanks (Paulet, 2011). Once females had begun spawning, the air- and water flow is turned off, allowing the negatively buoyant ova to settle on the bottom of the tank.

For the purpose of this study, unfertilized ova and sperm were obtained prior to fertilization to assess the efficacy of the respective chemicals to kill gametes. Unfertilized ova were collected from five random tanks housing one female each, and pooled for the purpose of this study. Once the ova had settled on the bottom of the tank (approximately 30 minutes), the ova were siphoned with seawater from the bottom of each female tank using a 10 mL serological pipette (Lasec Laboratories, South Africa) and transferred to a 250 mL Erlenmeyer glass flask (Lasec Laboratories, South Africa). Male gametes were collected by dipping a 250 mL glass beaker (Lasec Laboratories, South Africa) into three different male tanks, each containing five male animals to minimize the effect of individual variation (Visser-Roux, 2011). The collection of ova and sperm included seawater from the male and female tanks (storage medium).

In order to calculate the concentration of sperm collected, a volume of 1 mL of seawater containing sperm was transferred from the 250 mL glass beaker (sperm swim throughout the water column) into a 1.5 mL Eppendorf tube (Lasec Laboratories, South Africa) using a single channel pipette (Lasec Laboratories, South Africa). A measure of 1 µL formaldehyde (40%; Protea Chemicals, South Africa) was added to the same Eppendorf tube, using a single channel pipette (Visser-Roux, 2011). The addition of formaldehyde slows the swimming action of the sperm. A cover slip (1.5 x 1.5 cm; Lasec Laboratories, South Africa) was carefully placed over a Neubauer haemocytometer before the sperm were loaded into the Neubauer haemocytometer. An aliquot of 15 µL of sperm was then loaded into the Neubauer haemocytometer using a single channel pipette. Care was taken to avoid the formation of air bubbles when loading samples into the Neubauer haemocytometer chamber.

The concentration of sperm was calculated by counting the number of sperm within five blocks on each side (total of ten blocks) of the Neubauer haemocytometer (Hafez and Hafez, 2000). The average of sperm per block was calculated, by multiplying the average number of sperm per block by 50 000, results in the number of sperm in 1 mL of the 250 mL water sample collected from the male tanks. An equation ($C_1V_1 = C_2V_2$) was used to calculate the volume of the sample water that would contain a specific number of sperm (50 000 or 1 million), depending on the requirement for the exposure treatments (Visser-Roux, 2011).

The ova concentrations were calculated volumetrically by counting the number of ova per 1 mL using a microscope. The counts were repeated three times to verify the average number of ova per mL. The volume of sample water containing a specific number of ova (100 or 1 000) depending on the treatment requirement, was calculated by using the ova/mL calculated (Visser-Roux, 2011).

Ova and sperm were then stored in the original collection Erlenmeyer flak and glass beakers at 18°C in a refrigerated incubator (LM 570-R, MRC) while the experimental procedures were carried out (Visser-Roux).

*5.2.2.2 Collection of larvae and settlement larvae*

Larvae were obtained from fertilizations, at an optimal ratio of 1 000 ova to 50 000 sperm (Visser-Roux, 2011). The gametes for the fertilizations were obtained as described in Section 5.2.2.1.

A 10 mL syringe (Lasec Laboratories, South Africa) was used to add filtered seawater into 10 mL Nunc wells (Lasec Laboratories, South Africa). The calculated volume of water containing 50 000 sperm and 1 000 ova, was added to the prefilled Nunc wells using a single channel pipette (Lasec Laboratories, South Africa). Nunc wells were placed in an incubator (LM 570-R, MRC) at 18°C and incubated overnight. Hatch-out occurred 16 to 18 hours later.

The exposure of metamorphosed and settled larvae to chemical reagents was not performed. The removal of these animals for the trial was not allowed by the farm. Metamorphosized animals would have settled onto diatom plates, and detachment is unlikely. The possibility to expose five-day old larvae (pre-settlement tanks) became available during one of the trial days

Production protocol indicates that larvae are transferred larvae from the larval rearing tanks into 25 L buckets before they are added to settlement tanks. In order to obtain the settlement larvae for this study, a 250 mL glass beaker (Lasec Laboratories, South Africa) was immersed into the 25 L buckets, obtaining the settlement larvae.

*5.2.2.3 Collection of spat*

After a period of time in settlement tanks, spat are transferred to the weaning section. Here animals are housed in shallow plastic rectangular tanks at specific stocking densities. Spat obtained for this study were 1 cm in length. Spat were randomly removed from the black cones by slowly turning over the cone, and exposing the animals. Using a thumb and index finger the animals are slid off the plastic cone, placed onto a piece of mesh, and transferred to the lab for experimental procedures. Spat were removed in batches of nine animals, and only when they were required for chemical exposure.

### 5.2.3 Preparation of chemical reagents

Stock solutions were prepared by adding the concentrated chemical to filtered seawater (90 micron) at 18°C. A sachet of Biox™ (Immunovet, South Africa) was placed into a 12 L plastic bucket filled with filtered seawater, and left to dissolve for 30 minutes. The sodium hypochlorite (Protea Chemicals, South Africa) stock solution was prepared by adding 10 mL of concentrated sodium hypochlorite (Protea Chemicals, South Africa) to a 2 L glass beaker (Lasec Laboratories, South Africa) filled with filtered seawater. Chemicals were prepared at the start of each treatment day. Biox™ was stored in a black plastic 25 L bucket with a lid as chlorine dioxide is highly volatile, and sodium hypochlorite was stored in a 2 L glass beaker for the duration of the treatments. Chemicals were discarded after every treatment day; therefore the shelf life of the chemicals did not affect the potency of the chemicals.

**Table 5.1.** The volume of Biox™ and sodium hypochlorite stock solutions added to filtered seawater in a 250 mL glass beaker to prepare chemical concentrations required for the chemical exposure treatments.

| Chemical | Concentration (ppm) | Volume of sea water (mL) | Volume of chemical stock solution (mL) |
|---|---|---|---|
| Biox™ | 10 | 250 | 2 |
| | 50 | 250 | 10 |
| | 100 | 250 | 20 |
| Sodium hypochlorite | 10 | 250 | 0.2 |
| | 50 | 250 | 1 |
| | 100 | 250 | 2 |

After the sodium hypochlorite and Biox™ stock solutions were prepared, further dilutions were prepared in 250 mL glass beakers filled with filtered seawater (90 µm) at 18°C. Table 5.1 indicates the volume of stock solutions used to prepare the required concentrations. Test strips (Oxystix H/R, Immunovet) obtained from Immunovet (South Africa) were used to confirm the concentrations (ppm) of the sodium hypochlorite and Biox™ solutions during preparation. Test strips measure the free chlorine within the treatment water, indicating the concentration of the chemical reagents.

### 5.2.4 Experimental layout

*5.2.4.1 Preliminarily exposure protocol*

Preliminary exposure of sperm to chemical concentrations, ranging from 2.5 ppm, to 5 ppm or 10 ppm, indicated that concentration levels from 10 ppm and higher would be required to ensure an appropriate lethal or toxic effect. Similarly, preliminary exposure of ova indicated that concentrations higher than 50 ppm would be required to have a lethal effect.

**Table 5.2.** The experimental design of exposing five abalone (*Haliotis midae*) life stages to two chemical reagents; Biox™ and sodium hypochlorite.

| Life Stage | Treatment | | | |
|---|---|---|---|---|
| | Chemical | Concentrations (ppm) | Duration | Repeats |
| Sperm | Sodium hypochlorite | 10, 50 | 4, 6, 8, 10 and 12 minutes | X3 |
| | Biox™ | 10, 50 | 4, 6, 8, 10 and 12 minutes | X3 |
| Ova | Sodium hypochlorite | 10, 100 | 10 sec | X5 |
| | Biox™ | 10, 100 | 10 sec | X5 |
| Larvae | Sodium hypochlorite | 10, 50 | 4, 6, 8, 10 and 12 minutes | X3 |
| | Biox™ | 10, 50 | 4, 6, 8, 10 and 12 minutes | X3 |
| Settlement | Sodium hypochlorite | 100 | 4, 6, 8, 10 and 12 minutes | X3 |

| | | | | |
|---|---|---|---|---|
| Larvae | Biox™ | 10 | 4, 6, 8, 10 and 12 minutes | X3 |
| Weaning | Sodium hypochlorite | 10, 100 | 4, 6, 8, 10 and 12 minutes | X3 |
| | Biox™ | 10, 100 | 4, 6, 8, 10 and 12 minutes | X3 |

Based on the preliminary exposure to sperm, the concentrations ranging from 10 ppm to 100 ppm were subsequently chosen to perform the respective exposure experiments, as summarized in Table 5.2. A limited opportunity presented itself also to conduct an exposure treatment on five-day old larvae (pre-settlement tanks). A treatment concentration of 100 ppm at the five exposure times was chosen, on the basis that treatments of 10 ppm and 50 ppm had already been exposed to one-day old larvae.

### 5.2.5 Experimental protocol

*5.2.5.1 Determining the effect of chemical exposure on sperm viability*

Sperm were collected and concentrations were calculated using the equation $C_1V_1 = C_2V_2$, as described in Section 5.2.2.1. Nunc wells (Lasec Laboratories, South Africa) were filled, using a 10 mL syringe (Lasec Laboratories, South Africa) with either Biox™ (Immunovet, South Africa) or sodium hypochlorite (Protea Chemicals, South Africa) at either 10 ppm or 50 ppm, depending on the treatment. The exposure protocol of all sperm treatments was standardized.

The volume of collected water calculated to contain 1 million sperm, was added to pre-filled Nunc wells using a single channel pipette (Lasec Laboratories, South Africa). After sperm were added, concentrations were adjusted, as the addition of sperm diluted the concentrations in the Nunc wells. Approximately 0.7 mL of the chemical reagent was added for 50 ppm adjustments, and 0.3 mL was added for 10 ppm adjustments. The concentrations were continually tested with chlorine strips (Immunovet, South Africa). The sperm were left to react with the chemical reagents for the five exposure times of 4, 6, 8, 10 or 12 minutes. After each exposure time, a drop of eosin-nigrosin stain was added to the Nunc well and allowed to react with the solution for 1 minute. Three microscope slide smears were made from each treatment Nunc well (Lasec Laboratories, South Africa) by dipping one side of a microscope slide (Lasec Laboratories, South Africa) into the Nunc well and using a second microscope slide to evenly spread the liquid across the microscope slide. The slides were labeled and allowed to air dry.

The slides were assessed for live and dead staining of sperm under an inverted microscope (Olympus, IX 51) at 40x magnification. The Eosin-Nigrosin stain, stains dead sperm purple, while live sperm remain unstained (white). During slide assessment it was noted that all stained sperm were dead, therefore the slides were classified according to the type of damage that resulted from exposure of the sperm to the respective chemicals. The different categories are presented in Table 5.3.

**Table 5.3.** A description of the three categories of damage, used to assess the degree of morphological damage by exposure of sperm to Biox™ and sodium hypochlorite at 10 ppm and 50 ppm at five different time intervals.

| Category | Description |
|---|---|
| **Full** | A sperm with normal morphology, staining as dead |
| **Tail** | A sperm with a destroyed tail |
| **Head** | A sperm with only the head visible (completely detached tail) |

Two hundred sperm were counted per slide, with the sperm classified into one of three damage categories (Table 5.3), depending on the degree of damage observed. An average was calculated for the three slides per treatment and the categories were expressed as a proportion of the overall counts.

*5.2.5.2 Determining the effect of chemical exposure on ova viability*

Female gametes were collected and concentrations calculated as described in Section 5.2.2.1. A slide (Lasec Laboratories, South Africa) was placed on the microscope stage and a 4x magnification was used to assess the damage. A calculated volume of water containing 100 ova was placed onto the glass microscope slide using a single channel pipette (Lasec Laboratories, South Africa). A drop of sodium hypochlorite (Protea Chemicals, South Africa) or Biox™ (Immunovet, South Africa) at a concentration of 100 ppm was added to the ova on the microscope slide using a syringe (Lasec Laboratories, South Africa). Counts were done immediately after the drop of chemical reagent was added to the slide. During the preliminary exposure controls, a concentration of 100 ppm caused immediate damage (10 seconds); therefore further time intervals were not statistically analyzed. The ova were classified into one of the two categories described in Table 5.4 depending on the damage observed to the chorion. The number of ova in each group was expressed as a percentage (%) of the number of ova treated.

**Table 5.4.** A description of the two categories of damage, used to assess the damage by exposure of ova (*Haliotis midae*) to Biox™ and sodium hypochlorite at a concentration of 100 ppm at one time interval (10 seconds).

| Categories | Description |
|---|---|
| **Full** | The egg is morphologically normal (full chorion) |
| **Dissolved** | The egg chorion was visibly dissolved |

*5.2.5.3 Determining the effect of chemical exposure on larval viability*

Larvae were produced as described in Section 5.2.2.2. Nunc wells (Lasec Laboratories, South Africa) were prefilled with 10 mL of chemical reagent using a syringe (Lasec Laboratories, South Africa) for the exposure treatments. The water in the Nunc wells containing the larvae prepared in Section 5.2.2.2 was drawn with a single channel pipette (Lasec Laboratories, South Africa), and filtered through a 70 µm sieve (Lasec Laboratories, South Africa) to collect larvae, and discard any excess water. The sieve containing the larvae was placed directly into the prefilled Nunc well, exposing the larvae to one of the chemical reagents. Larvae were exposed to chemicals for five exposure times; 4, 6, 8, 10 or 12 minutes, and after each exposure time

was complete, the sieve was removed from the Nunc wells, and rinsed with filtered seawater using a syringe. During rinsing, by tilting the sieve, larvae were collected in one corner for ease of collection. A 0.5 mL single channel pipette was used to draw the larvae from the corner of the sieve and transferred into labeled 1.5 mL Eppendorf tube (Lasec Laboratories, South Africa) pre-filled with 1 mL of filtered seawater.

Larvae were transferred to clean 24-plate Nunc wells (Lasec Laboratories, South Africa), and the viability of the larvae assessed with a light microscope (Olympus IX 51) at 4x magnification. The larvae were recorded as live or dead according to their movement, and expressed as a percentage (%) of the total number of larvae counted.

*5.2.5.4 Determining the effect of chemical exposure on settlement larval viability*

Settlement animals were obtained by immersing 250 mL glass beakers (Lasec Laboratories, South Africa) into the settlement buckets, which were stocked at a commercial specific density for addition to settlement tanks. The density of larvae within these buckets was unknown; therefore in order to standardize the number of settlement larvae per treatment group, 1 mL of the collected water was used for each treatment.

Samples from the collected water was drawn up with a 1 mL pipette (Lasec Laboratories, South Africa) and filtered through a 70 µm sieve (Lasec Laboratories, South Africa) to collect larvae and discard excess water. Settlement larvae animals were exposed using the same method as described in Section 5.2.5.3 above.

Settlement larvae were transferred to clean 24-plate Nunc wells (Lasec Laboratories, South Africa) and the viability of the larvae assessed with a light microscope (Olympus, IX 51). The larvae were recorded as live or dead according to their movement, and expressed as a percentage (%) of the total number of larvae counted.

*5.2.5.5 Determining the effect of chemical exposure on spat*

Nunc wells (Lasec Laboratories, South Africa) were filled with 10 mL with sodium hypochlorite (Protea Chemicals, South Africa) or Biox™ (Immunovet, South Africa) at two concentrations described in Table 5.2. Nine spat were removed from weaning tanks as described in Section 5.2.3.3 for each of the treatments. Three animals were placed into a pre-filled (chemical) Nunc Well (Lasec Laboratories, South Africa) and exposed to chemicals for five exposure times; 4, 6, 8, 10 or 12 minutes After each exposure time was complete, animals were transferred to a glass container filled with clean filtered seawater. Animals were observed for five minutes in the glass container for changes in behaviour and mortality. Animals were recorded as either live or dead. Animals were considered dead if immobile, if they were floating or if excessive slime was produced. The number of dead spat were expressed as a proportion of the total number of animals exposed to the chemicals.

### 5.2.6 Statistical analysis

As there were unbalanced number of observations per group, the Least Square Means (LSM) were calculated to interpret the results. Data was analyzed using SAS for Windows (version 9.3; 2011). PROC GLM was used to analyze the data for all five life stages. Due to the unbalanced number of observations Type III sum of squares were calculated using PROC GLM in order to analyse the number of live and dead animals per life stage and to obtain the Least square means (LSM's) and Standard error of the mean (SEM) for all response variables viz. ova, sperm, larvae, settlement larvae and spat.

## 5.3 Results

### 5.3.1 The effect of chemical exposure on sperm viability

The results of this study showed that both Biox™ (Immunovet, South Africa) and sodium hypochlorite (Protea Chemicals) were effective in damaging sperm. No live sperm were present in the nigrosin-eosin smears, for any of the concentrations or duration of exposure. Therefore only classification of sperm according to the type of cell damage is presented.

The results indicated that both Biox™ and sodium hypochlorite caused the most significant morphological damage (head of sperm) at a concentration of 50 ppm ($P < 0.05$; Table 5.5). The chemical treatments did not differ significantly in their ability to damage sperm (0.85 ± 0.02 vs. 0.79 ± 0.02; $P > 0.05$; Table 5.5) at a concentration of 50 ppm.

The factorial analysis indicated that the interaction, between exposure time and chemical influenced the degree of damage when sperm were exposed to a concentration of 50 ppm, was significant ($P < 0.05$; Table 5.6).

**Table 5.5.** The influence of chemical and concentration on the viability of abalone (*Haliotis midae)* sperm (mean ± SEM), as quantified by means of proportion damage to the head.

| Chemical | Concentration (ppm) | Damage (Proportion head) | SEM |
|---|---|---|---|
| Biox™ | 10 | 0.09[a] | 0.02 |
| Sodium hypochlorite | 10 | 0.12[a] | |
| Biox™ | 50 | 0.85[b] | |
| Sodium hypochlorite | 50 | 0.79[b] | |

[a, b, c] Rows with different superscripts differ significantly ($P < 0.05$)

Table 5.6 shows the proportion head (the category indicating the most morphological damage to sperm through chemical exposure) observed when sperm were exposed to Biox™ and sodium hypochlorite at a concentration of 50 ppm over the five exposure times.

**Table 5.6.** The influence of chemical and duration of exposure on the viability of abalone (*Haliotis midae)* sperm (mean ± SEM), as quantified by means of proportion damage to the head.

| Treatment | | Mean Proportion head | SEM |
|---|---|---|---|
| **Duration (minutes)** | **Chemical** | | |
| 4 | Sodium hypochlorite | $0.72^{ab}$ | 0.04 |
| 6 | Sodium hypochlorite | $0.66^{a}$ | |
| 8 | Sodium hypochlorite | $0.86^{b}$ | |
| 10 | Sodium hypochlorite | $0.82^{ba}$ | |
| 12 | Sodium hypochlorite | $0.90^{b}$ | |
| 4 | Biox™ | $0.86^{b}$ | |
| 6 | Biox™ | $0.84^{ab}$ | |
| 8 | Biox™ | $0.84^{ab}$ | |
| 10 | Biox™ | $0.85^{ab}$ | |
| 12 | Biox™ | $0.89^{b}$ | |

$^{a, b, c}$ Rows with different superscripts differ significantly ($P < 0.05$)

The proportion of damaged heads observed over the five increasing exposure times were not significant ($P > 0.05$) when sperm were exposed to Biox™. The highest number of damaged heads was observed at a time of 12 minutes, 0.89 ± 0.04. The exposure of sperm to sodium hypochlorite indicated significant difference between an exposure time of 6 and 12 minutes (0.66 ± 0.04 and 0.90 ± 0.04; $P < 0.05$).

### 5.3.2 The effect of chemical exposure on ova viability

The interaction between the chemical and the concentration used was not significant ($P > 0.05$), and therefore only the main effects are discussed. The concentrations indicated significant differences ($P < 0.05$) in the damage to the ova. A chemical concentration of 100 ppm indicated an LSM of 109.2 ± 8.40 damaged chorion, while the concentration of 10 ppm had no effect on the chorion of the ova, all values were zero (Table 5.7).

**Table 5.7.** The influence of Biox™ and sodium hypochlorite exposure at two concentrations (10 ppm and 100 ppm) on the viability of abalone (*Haliotis midae*) ova (mean ± SEM), as quantified by means of damage to the chorion (mean ± SEM).

| Treatment | Damage (no chorion) | SEM |
|---|---|---|
| **Chemical** | | |
| Biox™ | 48.2[a] | 4.80 |
| Sodium hypochlorite | 61.0[a] | |
| **Concentration (ppm)** | | |
| 10 ppm | 0[b] | 4.80 |
| 100 ppm | 109.2[c] | |

[a, b, c] Rows with different superscripts differ significantly (P < 0.05)

The effects of Biox™ and sodium hypochlorite on the chorion damage were not significantly different (P > 0.05; Table 5.7).

The number of damaged chorion for the effect of the chemicals in Table 5.7 included all the zero values from the exposure of ova to a concentration of 10 ppm, this resulted in LSM's being halved. Table 5.8 shows the number of damaged ova (categorized by a damaged chorion) when ova were exposed to chemicals at a concentration of 100 ppm.

**Table 5.8.** The influence of Biox™ and sodium hypochlorite exposure at a 100 ppm concentration on the viability of abalone (*Haliotis midae*) ova, as quantified by damage to the chorion (mean ± SEM).

| Treatment | Damage (no chorion) | SEM |
|---|---|---|
| Biox™ | 96.4[a] | 8.90 |
| Sodium hypochlorite | 122.0[a] | |

[a, b, c] Rows with different superscripts differ significantly (P < 0.05)

The exposure of ova to sodium hypochlorite at Biox™ at a concentration of 100 ppm indicated a high damage to the chorion (122 ± 8.9 and 96.4 ± 8.9; Table 5.8), although the means observed for each of the chemicals did not differ significantly (P > 0.05).

### 5.3.3 The effect of chemical exposure on larvae viability

The effect of sodium hypochlorite and Biox™ on larval viability was influenced by three significant interactions (P < 0.05) namely; the interaction between the chemical reagents and the concentrations (P < 0.05), the concentrations and contact time (P < 0.05) and the interaction between the chemical reagents and the contact time (P < 0.05), therefore only the significant interactions are discussed.

Table 5.9 shows the percentage (%) mortality of larvae observed when larvae were exposed to Biox™ and Sodium hypochlorite at concentrations of 10 ppm and 50 ppm.

**Table 5.9.** The influence of Biox™ and sodium hypochlorite at two concentrations (10 ppm and 50 ppm) on the viability of abalone (*Haliotis midae*) larvae (mean ± SEM), as quantified by means of percentage (%) larval mortality.

| Treatment | | | |
|---|---|---|---|
| **Concentration (ppm)** | **Chemical** | **Percentage Mortality (%)** | **SEM** |
| 10 | Biox™ | 96.2[a] | 2.16 |
| 10 | Sodium hypochlorite | 50.2[b] | 2.07 |
| 50 | Biox™ | 100[a] | 2.07 |
| 50 | Sodium hypochlorite | 94.5[a] | 2.16 |

[a, b, c] Rows with different superscripts differ significantly ($P < 0.05$)

The highest percentage (%) mortality was observed when larvae were exposed to Biox™ at a concentration of 50 ppm (100% ± 2.07), this did not differ significantly ($P > 0.05$) from the percentage (%) mortality observed when larvae were exposed to sodium hypochlorite at 50 ppm (94.5% ± 2.16), and Biox™ at a concentration of 10 ppm (96.2% ± 2.16). The exposure of larvae to sodium hypochlorite at a concentration of 10 ppm (50.2% ± 2.07) indicated a significantly lower percentage (%) mortality ($P < 0.05$) compared to the other three treatments.

Table 5.10 indicates the percentage (%) mortality for the significant interaction between concentration and exposure time ($P < 0.05$) of both chemicals.

**Table 5.10.** The influence of chemical concentration (10 ppm and 50 ppm) and duration of exposure on the viability of abalone (*Haliotis midae*) larvae (mean ± SEM), as quantified by means of percentage (%) larval mortality.

| Treatment | | Percentage Mortality (%) | SEM |
|---|---|---|---|
| **Concentration (ppm)** | **Duration (minutes)** | | |
| 10 | 4 | 56.2[a] | 3.28 |
| 10 | 6 | 66.6[ab] | |
| 10 | 8 | 71.4[ac] | 3.63 |
| 10 | 10 | 87.0[cd] | 3.28 |
| 10 | 12 | 84.9[cd] | |
| 50 | 4 | 87.5[d] | |
| 50 | 6 | 98.2[d] | |
| 50 | 8 | 100[d] | |
| 50 | 10 | 100[d] | |
| 50 | 12 | 100.7[d] | 3.63 |

[a, b, c] Rows with different superscripts differ significantly ($P < 0.05$)

The percentage (%) mortality increased over time when larvae were exposed to a concentration of 10 ppm. The exposure of larvae to a concentration of 50 ppm resulted in a 100% mortality of larvae for three time periods (8, 10 and 12 minutes). No significant differences ($P > 0.05$) were observed in the mortality of larvae, when larvae were exposed to a lower concentration (10 ppm) for extended exposure times (10 and 12 minutes) against the exposure of larvae to a higher concentration (50 ppm) at shorter exposure times (4 and 6 minutes).

Table 5.11 shows the exposure of larvae to Biox™ and sodium hypochlorite over five exposure times. The percentage (%) mortality significantly increased ($P < 0.05$) with time when larvae were exposed to sodium hypochlorite (Table 5.11).

**Table 5.11.** The influence of chemical used and duration of exposure on the viability of abalone (*Haliotis midae*) larvae (mean ± SEM), as quantified by means of percentage (%) larval mortality.

| Treatment | | Percentage Dead (%) | SEM |
|---|---|---|---|
| **Chemical** | **Duration (minutes)** | | |
| Biox™ | 4 | 95.9[a] | 3.28 |
| Biox™ | 6 | 100[a] | 3.28 |
| Biox™ | 8 | 99.2[a] | 3.63 |
| Biox™ | 10 | 96.1[a] | 3.28 |
| Biox™ | 12 | 99.5[a] | 3.28 |
| Sodium hypochlorite | 4 | 47.7[b] | 3.28 |
| Sodium hypochlorite | 6 | 64.8[c] | 3.28 |
| Sodium hypochlorite | 8 | 72.2[d] | 3.28 |
| Sodium hypochlorite | 10 | 90.9[a] | 3.28 |
| Sodium hypochlorite | 12 | 86.1[a] | 3.63 |

[a, b, c] Rows with different superscripts differ significantly ($P < 0.05$)

Exposure of larvae to Biox™ resulted in high percentage (%) mortalities at all five exposure times; these percentages did not differ significantly ($P > 0.05$).

### 5.3.4 The effect of chemical exposure on settlement larvae viability

The interaction between the chemical and the time of exposure was not significant ($P > 0.05$), and therefore only the main effects are discussed (Table 5.12).

**Table 5.12**. The influence of chemical concentration on the viability of abalone (*Haliotis midae*) settlement larvae (mean ± SEM), as quantified by means of percentage (%) settlement larvae mortality.

| Chemical | Percentage Dead (%) | SEM |
|---|---|---|
| Biox™ (100 ppm) | 91.6[a] | 1.72 |
| Sodium hypochlorite (100 ppm) | 98.4[b] | 1.72 |

[a, b, c] Rows with different superscripts differ significantly (P < 0.05)

The percentage (%) mortalities observed when larvae were exposed to sodium hypochlorite and Biox™ at a concentration of 100 ppm were significantly different (P < 0.05). Sodium hypochlorite indicated a higher percentage (%) mortality (98.4% ± 1.72) than Biox™ (91.6% ± 1.72; Table 5.12).

### 5.3.5 The effect of chemical exposure on spat viability

The exposure of spat to Biox™ and sodium hypochlorite was significantly influenced by the interactions between the chemical reagent and the concentration used (P < 0.05), as well as the interaction between the concentration used (10 ppm and 100 ppm) and the five exposure times (4, 6, 8, 10 or 12 minutes; P < 0.05). The interaction between the chemical and the exposure time was not significant (P > 0.05) and are thus not shown.

The exposure of spat to sodium hypochlorite and Biox™ at a concentration of 100 ppm resulted in a significantly higher proportion mortalities (P < 0.05; 0.42 ± 0.04; 0.89 ± 0.04) compared to the exposure at concentration of 10 ppm (0 ± 0.04; 0.09 ± 0.041; Table 5.13).

**Table 5.13.** The influence of chemical concentration on the viability of abalone (*Haliotis midae*) spat (mean ± SEM), as quantified by means of proportion dead spat.

| Treatment | | Proportion dead | SEM |
|---|---|---|---|
| Chemical | Concentration (ppm) | | |
| Biox™ | 10 | 0.09[a] | 0.04 |
| Sodium hypochlorite | 10 | 0[a] | |
| Biox™ | 100 | 0.89[b] | |
| Sodium hypochlorite | 100 | 0.42[c] | |

[a, b, c] Rows with different superscripts differ significantly (P<0.05)

The proportion of spat mortalities observed between Biox™ at a concentration of 100 ppm (0.89 ± 0.04) was significantly higher than the proportion of spat mortalities (0.89 ± 0.04) observed when spat were exposed to sodium hypochlorite at a concentration of 100 ppm (0.42 ± 0.04; Table 5.13).

The exposure of spat to a chemical concentration of 10 ppm indicated no significant difference in the proportion mortalities observed over the five exposure times (P > 0.05). The exposure of spat to a

concentration of 100 ppm indicated significant increases in the proportion mortalities over time ($P < 0.05$; Table 5.14).

**Table 5.14.** The influence of chemical concentration and duration of exposure on the viability of abalone (*Haliotis midae*) spat (mean ± SEM), as quantified by means of proportion dead spat.

| Treatment | | Proportion dead | SEM |
|---|---|---|---|
| **Concentration (ppm)** | **Duration (Minutes)** | | |
| 10 | 4 | $0.06^{a}$ | 0.07 |
| 10 | 6 | $0^{a}$ | |
| 10 | 8 | $0^{a}$ | |
| 10 | 10 | $0.06^{a}$ | |
| 10 | 12 | $0.11^{ac}$ | |
| 100 | 4 | $0.39^{cd}$ | |
| 100 | 6 | $0.61^{bd}$ | |
| 100 | 8 | $0.61^{bd}$ | |
| 100 | 10 | $0.83^{b}$ | |
| 100 | 12 | $0.83^{b}$ | |

$^{a, b, c}$ Rows with different superscripts differ significantly ($P < 0.05$)

The highest proportion mortality was observed at time 10 and 12 minutes (0.83 ± 0.07: 0.83 ± 0.07; Table 5.14), but was not significant ($P > 0.05$). The proportion mortalities observed when spat were exposure to a concentration of 100 ppm at a time interval of 4 min (0.39 ± 0.07), did not differ significantly ($P > 0.05$) to when spat were exposed to a concentration of 10 ppm at a time interval of 12 minutes (0.11 ± 0.07; Table 5.14).

## 5.4 Discussion

This study investigated the potential of Biox™ and sodium hypochlorite to be used as chemical biocontainment agents to contain all respective life stages of *Haliotis midae* (HM) in commercial production systems. Limited information is available on chemical containment methods in the aquaculture industry' most chemical applications currently are used as medicines (including vaccines), antifoulants and disinfectants (Costello *et al.,* 2001).

The effect of the chemical reagents on the mortality observed varied for each of the life stages. A 100 % mortality rate was observed when sperm were exposed to Biox™ and sodium hypochlorite at concentrations of 10 ppm and 50 ppm for all five exposure times (2, 4, 6, 8, 10 or 12 minutes).

The exposure of ova to sodium hypochlorite and Biox™ at a concentration of 100 ppm had an immediate dissolving effect on the chorion (100% and 81.5%, respectively). Sperm and ova can therefore be exposed to a chemical concentration of 100 ppm of either chemical for a short exposure time to achieve the required effect on the gametes.

The exposure of larvae to sodium hypochlorite resulted in significantly higher mortalities when compared to Biox™ at a concentration of 100 ppm (98.4% and 91.6% respectively). The time of exposure had no significant effect on the mortalities observed in settlement larvae treatments.

The mortalities observed in one-day old larvae were influenced by significant interactions between the chemical, the concentration, as well as the time of exposure. The increase in the number of mortalities observed was directly related to the increase in the time of exposure, when one-day-old larvae were exposed to sodium hypochlorite. This however, was not observed in the exposure of one-day old larvae to Biox™, which did not did not show significant differences in the percentage (%) mortality over the five exposure times (95.9, 100, 99.2, 96.1 or 99.5%). It was also noted that percentage mortalities did not significantly differ between treatments of high concentrations (50 ppm) at shorter exposure times (4 minutes) and treatments of lower concentrations (10 ppm) at a longer exposure time (12 minutes).

Significantly higher proportion mortalities were observed when spat were exposed to higher concentrations of Biox™ (100 ppm; 0.89) compared to the exposure of spat to sodium hypochlorite of the same concentration (100 ppm; 0.42). The exposure of spat to higher concentrations (100 ppm) and higher contact times (10 and 12 minutes) indicated the highest, identical, rates of proportion mortality of 0.83. Visser-Roux (2011) conducted a study testing the toxicity of dimethyl sulfoxide (DMSO) on fertilized ova, and concluded that an increase in morphological abnormalities was directly correlated to the increase in the concentration of the dimethyl sulfoxide exposed to the fertilized ova. This was similarly seen when all life stages were exposed to increasing concentrations of the same chemicals. Cytotoxicity tests were done using various cryoprotectants for cryopreservation on molluscs, which indicated that larvae of starfish, *Asterina miniata*, Pacific oyster, *Crassostrea gigas*, white prawn, *Penaeus indicus,* and sea urchin, *Evechinus chloroticus*, were more resistant to the effects of the chemicals than fertilized ova or embryos, which was thought to be due to the larval membrane being less permeable (Newton 1996; Smith *et al.,* 2001; Hamaratog□lu *et al.,* 2005; Adams *et al.,* 2006; Visser-Roux, 2011).

As a chemical containment method, the exposure of HM life stages to higher concentrations of chemicals at shorter contact periods would be optimal within a hatchery. Commercial production systems are operated as a flow-through system with large volumes of water continuously flowing through the hatchery. Even though lower concentrations have shown to be as effective at longer contact intervals, this would be a less feasible method, as the relatively high flow rate of a hatchery system would not allow for an extended contact time.

In the laboratory treatments tested in this study, each HM life stage was isolated, and exposed separately to the chemical reagents. Flow rates of each housing section was not taken into consideration when the

exposures were tested. Short exposure times concerning the administration of the chemicals in a flow through system is desirable, as flow rates are high and will dilute concentrations once chemicals have been administered into the system. Therefore,, using the flow rates of each of the housing sections, it can be calculated how much chemical needs to be administered into the effluent water of each life stage in order to induce mortalities to animals.

Chemicals administered in aquaculture are most commonly added to a chamber or sump where the effluent water passes through (Wong and Van Eenennaam, 2008). Effluent water flows into the sump or chamber where the chemical interacts with the effluent water for a given amount of contact time, before being released into the environment. The use of a chemical chamber however would require integration into the current system and therefore would be costly to implement. Also, common practices in the use of chemical chambers or sumps require the water to be treated before being released back into the environment (Wong and Van Eenennaam, 2008), but as Chlorine dioxide (Biox™) and sodium hypochlorite are said to have very little harmful effect on the environment (Simpson *et al.,* 1993; EU, 2007), the restoration of water quality may not be necessary. Chlorine dioxide (Biox™) has a high volatility, and can lose between 7 to 30% of its effectiveness depending on its concentration and time of exposure. This means that dilutions prepared for stock solutions cannot be assumed to be quantitative (Benarde, 1965). Hypochlorites also decrease in strength over time in storage. Johnson and Remeikis (1965) found that a 5.25% (525 000 ppm) sodium hypochlorite solution remained stable over a 10-week period, but a 1% (1 000 ppm) sodium hypochlorite solution remained stable for only one week. The instability, and short shelf life of the chemicals may affect the management, and use of the chemicals, as the process could become expensive and labour intensive if new chemical stock solutions must constantly be made. Research on other chlorine-based chemicals with higher shelf life may be beneficial to the use of chemical containment.

The use of a Dosatron (Paulet, 2011), where a fixed concentration of a chemical is continually administered into the flow through system should be investigated. The Dosatron system has previously been used to administer medications in the livestock industry (Dosatron, USA, 2013). The use of a Dosatron, and other methods of chemical administration using the chemical concentrations used in this study should be further investigated, as they have shown effective mortalities in the life stages of an abalone hatchery. The investigation on effective chemical application methods must take the flow rates of the hatchery production unit into account.

## 5.5 Conclusion

The objective of this study was to investigate the potential of Biox™ and sodium hypochlorite to be used as a chemical method of containment in an abalone hatchery production system.

The exposure of HM life stages to Biox™ and sodium hypochlorite resulted in high mortalities throughout all life stages (sperm, ova, larvae, settlement larvae and spat), with the higher concentrations of chemical reagents together with the longer contact times causing the most extensive damage.

From the results it is recommended that higher concentrations of chemicals be used at shorter contact intervals in a continuous flow through production system, as this would be more practical based on high flow rates, which will dilute the concentrations of the chemicals once in contact with the effluent water. Future research should include investigation into appropriate methods of chemical administration, taking flow rates of the production unit into account as well as investigating chemicals with a longer shelf life, before chemical containment can be considered as a viable method of containment in an abalone hatchery production unit.

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# Chapter 6

# General conclusions and recommendations

*Haliotis midae* (HM) is the only abalone species that is commercially produced in South Africa. Current production protocols take three to five years for animals to reach to a marketable size of 70 - 100 grams (Hahn, 1989). Current research, including the field of biotechnology, is focusing on the improvement of production traits such as feed conversion rate (FCR), but more specifically on improving the growth rate of abalone (Galli, 2002; Devlin *et al.,* 2006*;* Wong and Van Eenennaam, 2008; Visser-Roux, 2011; Arai and Okumura, 2013). Biotechnologies pertaining to increased growth include transfections with growth genes and the induction of triploidy (Galli, 2002). Triploid induction is the manipulation of the genome of the animal, resulting in induced animals retaining a third set of chromosomes, which may render the treated animals sterile. This induced sterility can potentially allow animals to partition energy and protein into growth rather than gonad differentiation, development, and function (Allen *et al.,* 1986; Beaumont and Fairbrother, 1991; Zhang *et al.,* 1998; Ruiz-Verdugo *et al.,* 2000; De Beer, 2004; Piferrer *et al.,* 2009). Partitioning of energy and protein into growth rather than reproduction will potentially accelerate the growth rate of animals in commercial production systems, thus decreasing the time to harvest, which in turn will result in the more cost-effective production of abalone. Sterile triploid animals would also not experience typical side effects on their meat quality caused by sporadic spawnings, which can be triggered by stress during transport (Brake *et al.,* 2004). Therefore farms will be able to produce a quality product all year round, even during the spawning season (Brake *et al.,* 2004).

The use of biotechnologies such as gene transfection and triploid induction to improve the profitability of commercial abalone production systems creates a concern over the possible interaction between genetically modified (GM) farmed and wild abalone, especially if the transgenic abalone have an ability to outcompete the natural wild stocks through an enhanced fitness, the introduction of parasites, and altering the genetic diversity of wild stocks (Muir and Howard, 1999; Maclean and Laight, 2000; Hawkins and Jones, 2002; Theil *et al.,* 2004; Devlin *et al.,* 2006; Hu *et al.*, 2007). These concerns have highlighted the need for the development and implementation of containment methods that will be able to effectively contain GM and selectively bred diploid abalone genotypes.

The risks associated with the potential genetic impact of transgenic and selectively bred HM (i.e. both triploid and diploid individuals), on natural wild stocks has not yet been qualified and quantified for the South African industry due to the fact that the industry is relatively young when compared to other livestock industries (Theil *et al.,* 2004). Such risks can, however, be minimized through the effective use of containment methods (Rasmussen and Morrissey, 2007; Wong and Van Eenennaam, 2008). Containment strategies can include one or a combination of mechanical methods, which can include barriers and filters; biological methods, where triploid induction is an example; and lastly chemical methods, where organisms are exposed to chemicals that have a lethal or toxic effect. With the use of chemical containment, it is important to use an

agent that is environmentally friendly, and will not result in any ecological threat to the natural environment it will be released into.

To date, no containment strategies have been formulated for commercial abalone production systems under South African farming conditions. Farms have two sections, i.e. a grow-out section and a hatchery, with the latter including separate broodstock, larvae, settlement and weaning areas. Due to size and behavioural differences of the life stages, tailor-made containment methods will be required for each housing section. The study therefore investigated the potential of biological, mechanical and chemical containment methods to contain HM in a continuous flow-through production system. Biological methods involved the use of triploid induction as a potential means to render HM sterile, thus incapable of interbreeding with wild stocks. Mechanical methods involved the use of polypropylene filters and obstructive materials (Astroturf, carpet and Velcro) to contain HM, and the chemical methods investigated the suitability of sodium hypochlorite and Biox™ to contain HM. The sections below will present the major findings, and also make recommendations toward the incorporation and implementation of the findings in commercial abalone production systems in South Africa.

### Triploidy as a biological containment method of abalone (*Haliotis midae*)

The objective of this part of the study was to investigate the influence of triploid induction on gonad development and function in HM in order to evaluate the use of triploidy as a method of biological containment.

The results indicated that the gametogenic development of male and female triploid HM was negatively affected by the exposure of fertilized embryos to the hydrostatic pressure induction method. The gonads of the triploid male and female HM displayed significant abnormal development. Triploid male gonads were characterized by a small number of immature gametes, and a complete lack of mature gametes, compared to diploid male gonads, where normal mature spermatozoa were observed in the lumens. Triploid female gonads were characterized by the occurrence of a small number of mature gametes, with the bulk of the ovary lumen being occupied by immature gametes. Diploid female gonads were characterized by the presence of normal mature ova in the ovary lumen. Induced diploid male and female HM also indicated arrested gonad development, which could indicate the occurrence of mosaics within the treatment group.

It was found that even after a prolonged conditioning period triploid HM did not spawn following induction, therefore gametes for the fertilization trials were obtained using a biopsy technique. Fertilization results indicated that triploid sperm were not able to fertilize diploid ova. Low numbers of abnormal fertilizations (0.01%) were observed when induced diploid sperm were used to fertilize diploid ova. Histological assessments further classified male triploid HM as having only immature gametes, which would explain the result that triploid male animals were unable to fertilize normal diploid ova.

Although this study indicates that abnormal development occurred in triploid HM and no fertilizations were observed, the limited number of triploid animals available for the gametogenesis development and the fertilization studies has however affected the statistical validity and significance of the findings. A number of tags were lost during the initial genotype verification phase of the study, and therefore some of the genetically verified triploid animals could not be identified. True and complete sterility which is required for triploidy to be considered as a reliable method of biological containment of GM abalone in commercial systems could, however, not be confirmed as some level of mature gametes were observed in the triploid HM males and females.

*The following recommendations are to be considered in order to improve the assessment of triploidy as a biological containment method in HM:*

- Implementation of a more effective tagging method.
- Further research using bigger sample sizes in order to determine the age at which the induction will be most effective in creating true sterile HM individuals.
- Future research to determine the efficacy of the different triploid induction techniques to induce true sterility in HM.
- Further research on the stage at which spermatogenesis and oogenesis development is arrested in male and female HM.
- Future research on the possible effect that induction method has on the endocrine system. This may lead to an alternative method to manipulate the suppression of gametogenesis completely.
- Future research on the genetic verification and occurrence of mosaic animals.
- Future research on the effect of conditioning of spawning activity in mature triploid animals.

## Mechanical containment of abalone (*Haliotis midae*)

This part of the study investigated the efficacy of 100 µm filter bags placed in the commercial hatchery system to contain male and female gametes, larvae, and settlement larvae, as well as the use of obstructive materials (AstroTurf, carpet and Velcro) to impede the movement of spat from the housing units during the weaning stage. Abalone farms in South Africa consist of flow-through systems, which present the opportunity for the abalone to “escape” their housing, and be introduced back into the wild. Current on-farm containment methods include banjoe sieves (120 µm) that are incorporated into the larvae section, and 0.3cm mesh covering placed over the standpipes that collect the effluent water from the weaning section.

In this study, the 100 µm polypropylene filter bags could not effectively contain female broodstock gametes, as ova were observed in the effluent water samples. The average number of ova observed in effluent water samples increased as the female densities increased per tank increased, which was expected. Absolute containment was observed when the 100 µm polypropylene filter bags were evaluated for the containment of

one-day old and settlement larvae. The current use of a 120 µm banjoe sieve employed by the farms also proved to be an effective method of mechanical containment for the larvae section. The study indicated that the 100 µm filter bags are a feasible option for the first two days of the settlement stage, as no larvae were recorded in the effluent water samples after day three. Although the filter bags showed to be highly effective to contain HM in a small scale laboratory testing, it may not be ideal for a larger scale commercial abalone production due to the high flow rates that need to be accommodated, which put demands on the maintenance, amount and cleaning of the filter bags.

No statistical significant differences were observed in the efficacy of the different obstructive materials to contain spat over a 48-hour period, although it is recommended that the assessment should be performed over a longer period of time.

*The following recommendations are to be considered in order to improve the efficacy of mechanical containment in an abalone hatchery:*

- Further investigation into the use of a sand filter in the broodstock room to contain male and female HM gametes.
- Investigation into the labour intensity of using a filter bag on the first two days during the settling of larvae.
- Further investigation on the use of obstructive materials to impede the movement of spat – materials need to be evaluated over a longer period of time.
- Future investigation on the use of a sand filter in the broodstock housing section.
- Future research on the possibility of implementing a sand filter into an existing open continuous flow through abalone production system at points of major linkage in the drainage system.
- Future research on the probability of escaped larvae to settle in the surrounding natural habitat, and growing to sexual maturity.

### Chemical containment of abalone (*Haliotis midae*)

The third part of the study investigated the potential of sodium hypochlorite and Biox™ to chemically contain the respective life stages of HM. Sodium hypochlorite and Biox™ were selected as chemicals for they are considered to be environmentally friendly, and quickly degrade in an aqueous environment. Sodium hypochlorite is currently used in the footbaths of the bio-secure areas of abalone farms.

The exposure trials indicated that both sodium hypochlorite and Biox™ were effective chemical containment agents. The exposure of HM life stages to Biox™ and sodium hypochlorite resulted in high mortalities experienced in all life stages, with the higher concentrations of chemical reagents together with the longer contact times causing the most extensive damage in the respective life stages. Based on the findings of this study, it is recommended that higher concentrations (100 ppm) of chemicals be used at shorter exposure

times (4 minutes) in a continuous flow-through production system, as this would be more practical based on high flow rates, which will dilute the concentrations of the chemicals once in contact with the effluent water, thus losing efficacy.

*The following recommendations are to be considered in order to improve the efficacy of chemical containment in an abalone hatchery:*

- Further investigation on other environmentally friendly chemicals, which have longer storage times/shelf lives.
- Further research on the most appropriate method to introduce chemicals to the various sections of abalone production systems, which all differ in terms of the flow rate of the water supply.
- Further research on the use and feasibility of a chemical chamber and a Dosatron in a commercial hatchery production system

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