Optimization of gene transfer in *Haliotis midae* by means of polyplex mediation

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: *29 September 2010*

Summary

Haliotis midae is the most important aquaculture species in South Africa, with abalone farming contributing 80% of the Rand value of the aquaculture industry. Although genetic research has benefited the abalone industry, several issues still hinder increases in abalone production. Progress towards an increase in *H. midae* growth rate by utilizing conventional genetic studies and selective breeding has been relatively slow. Gene transfer has therefore become a plausible option to address this problem. Genes that code for certain desirable traits, such as increased growth rate, could be incorporated into the genome of commercial abalone.

The current study undertook the optimization of a chemically-mediated gene transfer technique using Polyethylenimine (PEI) as transfection reagent and fluorescent proteins as reporter genes. Before gene transfer could be undertaken, several complementary studies also needed to be undertaken due to the novel nature of the study. The autofluorescence of *H. midae*, the suitability of several *H. midae* tissues as targets for gene transfer and the cytotoxic effect of transfection reagents and selection antibiotics were assessed before gene transfer optimization could be attempted. Also, genes linked to an increase in growth rate were characterized for differential expression in different abalone age-groups to determine the suitability of these genes for incorporation into a homologous gene construct in future transfection studies.

The autofluorescence of ova, embryos and larvae were found to be comparable to that of the fluorescent reporter genes, EGFP and DsRed. A PCR-based transfection validation method was therefore employed to confirm the presence of internalized transgenes. It was established that sperm, ova, larvae and haemocyte cell culture were the most suitable target tissues for transfection. The transfection reagents, a 25kDa PEI and ExGen 500, were not cytotoxic to sperm, embryos and haemocyte cell cultures. The minimum lethal concentration of the selection antibiotics, neomycin and zeocin, was determined for larvae and haemocytes. After transfection treatment of sperm and fertilization of untreated ova, the presence of internalized transgenes could be verified for larvae. The presence of internalized transgenes could not be detected after transfection treatment of ova and larvae. Fluorescent flow cytometry and microscopy analysis of haemocytes could not detect the expression of the fluorescent reporter genes. Expression of two of the growth-related genes was found to differ between age-groups. The perlustrin gene was up-

regulated in older animals, while the insulin related peptide receptor gene was down-regulated in older animals. The third gene, a thrombospondin-1 precursor was stably expressed in all age-groups.

This study represents the first report of transfection studies carried out on *H. midae.* Future studies will benefit from the groundwork established in *H. midae* transfection.

Opsomming

Haliotis midae is die belangrikste akwakultuur spesie in Suid-Afrika met perlemoen boerdery wat 80% van die Rand waarde van die akwakultuur industrie bydrae. Alhoewel genetiese studies die perlemoen industrie ‘n hupstoot gegee het, is daar steeds sekere struikelblokke wat verdere toename in produksie verhoed. Vooruitgang ten opsigte van ‘n toename in *H. midae* se groei tempo deur gebruik te maak van konvensionele genetiese studies en selektiewe teling was tot dusver relatief stadig. Genetiese transformasie het daarom ‘n wesenlike alternatief geword wat moontlik hierdie probleem kan oplos. Gene wat kodeer vir sekere eienskappe, soos ‘n toename in groeitempo, kan in die genoom van kommersiële perlemoen inkorporeer word.

Die huidige studie het onderneem om ‘n chemies-gemedieerde genetiese transfeksie tegniek te optimiseer en van *Polyethylenimine* (*PEI*) as transfeksie reagens en fluoresserende proteine as verklikkers gebruik te maak. As gevolg van die oorspronklikheid van die studie moes verskeie bykomende ondersoeke ook aangepak word voordat genetiese transfeksie uitgevoer kon word. Die outofluoressensie van *H. midae*, die geskiktheid van verskeie *H. midae* teiken weefsels en die sitotoksiese effek van die transfeksie reagense en seleksie antibiotika is ondersoek voordat transfeksie uitgevoer is. Gene gekoppel aan ‘n toename in groeitempo is ook gekarakteriseer vir verskille in uitdrukking in verskillende perlemoen onderdoms-groepe om te bepaal of hierdie gene moontlik in ‘n homoloë geen konstruk ingesluit kan word vir toekomstige transfeksie studies.

Dit is gevind dat die outofluoressensie van ova, embrios and larwes vergelykbaar is met die fluoressensie van die verklikker proteïene, *EGFP* en *DsRed*. ‘n PKR-baseerde metode om die internalisering van die transgeen te kontroleer is daarom gebruik. Dit is vasgestel dat sperm, ova, larwes en haemosiete die mees geskikte teiken vir transfeksie sou wees. Die transfeksie reagense, ‘n 25kDa *PEI* en *Exgen 500*, is nie sitotoksies vir sperm, embrios of haemosiete nie. Die minimum dodelike konsentrasie van die seleksie antibiotika, neomycin en zeocin, is bepaal. Na transfeksie behandeling van sperm en bevrugting van onbehandelde ova, kon die teenwoordigheid van internaliseerde transgene bevestig word vir larwes. Die teenwoordigheid van internaliseerde transgene kon nie bevestig word na transfeksie behandeling van ova en larwes nie. Fluoressente vloei sitometrie en mikroskopiese analise kon nie die uitdrukking van die fluoressente verklikker

gene bevestig in haemosiete nie. Die uitdrukking van twee van die gene gekoppel aan groei het verskil tussen ouderdoms-groepe. Die perlustrin geen is meer uitgedruk in ouer diere terwyl die insulien geassosieerde peptied reseptor geen minder uitgedruk is in ouer diere. Die *thrombospondin-1* voorloper geen is stabiel uitgedruk in al die ouderdoms-groepe.

Hierdie studie verteenwoordig die eerste verslag van transfeksie studies uitgevoer op *H. midae*. Toekomstige studies sal baat vind by die grondslag wat deur hierdie projek gelê is.

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"Success is not a place at which one arrives but rather the spirit with which one undertakes and continues the journey." –Alex Noble

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List of Abbreviations

%: Percent

®: Registered Trademark

℃: Degrees Celsius

µg: Microgram

µl: Microlitre

µm: Micrometre

10X : Ten times

1X: One times

3’ : Three prime

5’ : Five prime

bp: Basepair

$CaCl_2$: Calcium chloride

CFP: Cyan Fluorescent Protein

cm: Centimetre

CMV: Cytomegalovirus

dH_2O: Distilled water

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

EDTA: Ethylenediamine Tetraacetic Acid

EGFP: Enhanced Green Fluorescent Protein

ELISA: Enzyme-Linked Immunosorbent Assay

F: Forward Primer

FBS: Fetal Bovine Serum

FITC: Fluorescein isothiocyanate

FSW: Filtered Sea Water

g: Gram

g: Gravitational force

GFP: Green Fluorescent Protein

GM: Genetically Modified

GMO: Genetically Modified Organism

GUS: Beta-Glucuronidase

H_2O: Hydrogen oxide (water)

HCl: Hydrogen chloride

kb: Kilobasis

kDa: Kilo-Dalton

L: Litre

LiCl: Lithium chloride

mg/ml: Milligram per millilitre

$MgCl_2$: Magnesium chloride

$MgSO_4$: Magnesium Sulphate

MIP: Molluscan Insulin-like Peptide

ml: Millilitre

mm: Millimetre

mM: Millimolar

NaCl: Sodium chloride

ng/µl: Nanograms per microlitre

ng/ml: Nanograms per millilitre

ng: Nanogram

nm: Nanometre

PCR: Polymerase Chain Reaction

PEI: Polyethylenimine

QTL: Quantitative Trait Loci

REST: Relative Expression Software Tool

RNA: Ribonucleic Acid

rRNA: ribosomal RNA

SDS: Sodium dodecyl sulfate

siRNA: small interfering RNA

SNP: Single Nucleotide Polymorphism

Taq: *Thermus aquaticus* DNA polymerase

TBE: Tris Borate EDTA

TE: Tris EDTA

™: Trademark

UV: Ultra Violet

v/v: volume per volume

w/v: weight per volume

YFP: Yellow Fluorescent Protein

ZAR: South African Rand

µM: Micromolar

1. Introduction

1.1. Abalone

Abalone are herbivorous, reef-dwelling, univalve, marine molluscs of the class Gastropoda and subclass Orthogastropoda. They belong to the suborder and order of Vetigastropoda and Archeogastropoda respectively, family Haliotidae, genus *Haliotis*. *Haliotis* exhibits a biphasic lifecycle consisting of a distinct pelagic larva and a benthic adult stage. This is an ancient lifecycle that most of the earliest phyla such as the pre-bilaterian and bilaterian exhibit [Jackson *et al*. 2002].

Abalone possess a convex shell with a spiral shape that covers the animal's delicate viscera and allows the muscular foot to protrude from the shell. The shell consists of calcium carbonate and aragonite platelets. These aragonite platelets of around 0.5µm are formed by successive nucleation of aragonite crystals [Lin and Meyers 2005]. The innermost layer of the shell has an iridescent mother-of-pearl colouring, called nacre. The shell's outermost layer is covered with algae, coral, sponges or other molluscs. A row of rounded openings that assist in respiration and waste removal are situated along the outer ridge of the shell [Fallu 1991].

The 56 recognized *Haliotis* species have a worldwide distribution [Geiger 2000]. Species differ in distribution, colour, size, growth rate and production value [Courtois de Vicose *et al*. 2007]. Currently several species are farmed for the main markets in China, Japan, Hong Kong, USA, Mexico, Korea and Europe [Oakes and Ponte 1996; Troell *et al*. 2006] (see Table 1.1).

Table 1.1: Not all abalone species are commercially farmed; the following species are the most commonly farmed species (Adapted from Fallu 1991).

Common name	Scientific name	Country of origin
Blacklip abalone	*H. rubra*	Australia
Ezo abalone	*H. discus hannai*	Japan
Greenlip abalone	*H. laevigata*	Australia
Ormer	*H. tuberculata*	Europe
Paua	*H. iris*	New Zealand
Perlemoen	*H. midae*	South Africa
Pinto abalone	*H. kamtschatkana*	North America
Red abalone	*H. rufescens*	Chile, California and Mexico
Small abalone	*H. diversicolor*	Japan and Taiwan

1.2. Abalone reproduction

1.2.1. Spawning

Abalone are seasonal dioecious broadcast spawners that eject large amounts of gametes through the fissures in their shell [Huchette *et al*. 2004]. Ripened gonads and the presence of other mature abalone are however not sufficient to induce spawning. Certain environmental cues are necessary. In nature, fluctuations in water temperature are the usual stimulus. In a farming environment, broodstock are induced to spawn by increasing the environmental stress on the animal. This is done by temperature shock, air exposure, exposure to ultra-violet irradiated water, addition of hydrogen peroxide or a combination of these. After successful induction, spawning takes place within a few hours [Fallu 1991]. In a commercial setting, a spawning event is the start of the production process where abalone proceed from fertilized larvae in the hatchery to settled spat, micro-algae feeding juveniles, macro-algae feeding juveniles and the grow-out phase from where animals are harvested for production (see Figure 1.1) [Fallu 1991].

1.2.2. Sperm

Reports on abalone sperm size indicate sperm to range from 32.6µm to 48.8µm depending on the species [Grubert *et al*. 2005]. Sperm is released at regular intervals during spawning at a rate of $5.3x10^7$ sperm/second with males having been recorded to emit 10^{12} sperm cells at a spawning event (*H. laevigata*) [Babcock and Keesing 1999; Grubert 2005]. This results in high sperm concentrations in the surrounding areas when

hydrodynamic conditions are calm [Huchette *et al*. 2004]. Chemical signals between sperm and ova play a pivotal role in fertilization success. It has been noted that sperm in the proximity of live ova move faster while orientating themselves towards the ova. Riffell *et al*. (2002) elucidated L-tryptophan to be the key sperm attractant, mediating activation and chemotaxis present in *H. rufescens* ova. This mode of attraction is especially important in turbulent aquatic environments, where sperm and ova are easily separated by water movements.

1.2.3. Ova

Abalone ova are negatively buoyant with a size of approximately 0.2mm, a green colour and enveloped by a jelly-like vitelline layer [Swanson *et al*. 2001]. The size of ova depends foremost on the species, but also the genotype of the individual as well as the reproductive status [Baker and Tyler 2001]. Female abalone have been recorded to release between $5.9x10^6$ to $8.2x10^6$ ova per spawning event (*H. rubra, H. laevigata*) [Babcock and Keesing 1999; Litaay and Da Silva 2001]. The number of ova emitted is however also related to the weight of the individual female [Baker and Tyler 2001]. Ova contain the nutrients that nourish the lecithotrophic larvae until they undergo metamorphosis and can start feeding off micro-algae [Huchette *et al*. 2004].

1.2.4. Fertilization

Fertilization success of abalone is dependent on sperm concentration, the sperm-ova ratio, the age of gametes and the time sperm and ova are in contact. Baker and Tyler (2001) found that increasing the sperm concentration increased the fertilization success up to a concentration of 10^6 sperm/ml in *H. tuberculata.* Leighton and Lewis (1982) also found 10^6 sperm/ml to be most efficient for fertilization in *H. rufescens*, *H. corrugata*, *H. fulgens* and *H. sorenseni* (see Table 1.2).

Table 1.2: The optimum sperm concentration of several abalone species (Adapted from Baker and Taylor 2001).

Species	Sperm concentration for optimum fertilization	Reference
H. asinina	$5x10^3$ - 10^5 sperm/ml	Encena *et al*. (1998)
H. corrugata	10^5 - 10^6 sperm/ml	Leighton and Lewis (1982); Mill and McCormick (1989)
H. discus hannai	10^5 - 10^6 sperm/ml	Gao *et al*. (1990)
H. diversicolor	$2x10^4$ sperm/ml	Fallu (1991)
H. fulgens	10^5 - 10^6 sperm/ml	Leighton and Lewis (1982); Mill and McCormick (1989)
H. laevigata	$2x10^5$ sperm/ml; 10^4 - 10^6 sperm/ml	Fallu (1991); Babcock and Keesing (1999)
H. rubra	$2x10^5$ sperm/ml	Fallu (1991)
H. rufescens	10^5 - 10^6 sperm/ml	Leighton and Lewis (1982); Mill and McCormick (1989)
H. sorenseni	10^6 sperm/ml	Leighton and Lewis (1982)
H. tuberculata	10^5 - 10^6 sperm/ml	Clavier (1992); Baker and Tyler (2001)

Reports on the optimum ratio of sperm to ova have yielded varied results. Wang *et al*. (2004) reported the ratio to be 1:10 000 in *H. discus hannai*. Baker and Tyler (2001) found a ratio of between 100:1 and 500:1 to be the optimum for fertilizations for *H. tuberculata*, with a ratio of less than 30:1 showing a decrease in fertilization success. A ratio of more than 500:1 exhibited a marked decrease in fertilization success, most likely due to polyspermy; where the high concentration of sperm and subsequent sperm lysin destroys the vitelline layer of ova and result in the degeneration of ova and abnormal fertilizations with more than one sperm cell [Grubert *et al*. 2005].

Optimum fertilization is reported to take place within 30 minutes of spawning of the ova, with no fertilization taking place after 2.5 hours [Baker and Tyler 2001] (see Table 1.3).

Table 1.3: The maximum time of sperm viability in some bivalve and abalone species (Adapted from Baker and Taylor 2001).

Species	Maximum sperm viabilty	Sperm concentration	Reference
Cerastoderma edule (bivalve)	4-8 hours	10^5 sperm/ml	André and Lindegarth (1995)
Mytilus edulis (bivalve)	More than 5 hours	10^6 sperm/ml	Levy and Couturier (1996)
H. asinina	More than 2 hours	10^5 sperm/ml	Encena *et al*. (1998)
H. tuberculata	2.5 hours	10^6 sperm/ml	Baker and Tyler (2001)

After fertilization, cell division ensues giving the developing bundle of cells the classic morula, blastula and gastrula appearance (see Figure 1.1). The gastrula matures into the egg membrane encased trochophore larvae. As soon as development has proceeded to a suitable stage, the trochophore escapes the egg membrane to hatch as a free-swimming veliger larvae from which the adult characteristics develop after settling [Fallu 1991].

1.2.5. Larvae

In 1952, Ino characterized larval development for *H. discus hannai*. This has served as a guideline for the characterization of the larval stage of other *Haliotis* species as the larval stage of most haliotid species are fairly analogous. The larval stage can be characterized in 39 distinct stages (see Courtois de Vicose *et al*. 2007 for a comprehensive characterization). The lecithotrophic abalone larvae hatch within 24 hours of fertilization. The length of the larval stage, which is from fertilization to the formation of the third tubule on the cephalic tentacles, ranges between species and is influenced by environmental conditions. The larval stage can range from 4 to 15 days in different species and under different conditions [McShane 1992]. Generally the 90º stage of torsion and the development of the foot and operculum occur 3 days after fertilization. Full torsion, when the larvae establish muscular control of the operculum and permanent shell, only occurs 8 to 10 days after fertilization [Nash 1991]. The larvae are mobile by means of cilia and are itinerant in natural population by means of water currents; ensuring that larvae are dispersed and genetic heterogeneity is maintained as well as minimizing competition between parent and offspring [Huchette *et al*. 2004].

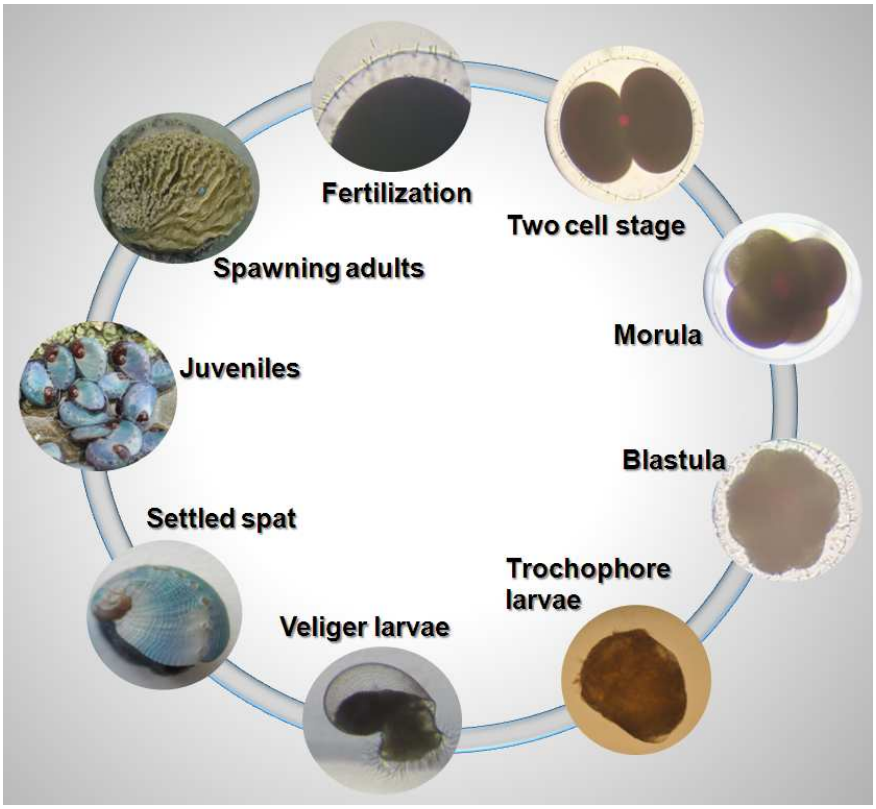

Figure 1.1: Abalone undergo metamorphosis from a benthic lifestyle as larvae to a pelagic lifestyle once settling occurs. *Haliotis midae* matures and starts spawning at approximately four years of age in a commercial environment.

1.3. Mollusc cell culture

Establishing a permanent and proliferative cell line from marine invertebrate tissues has been problematic and no such line exists as yet. Numerous mammalian and even insect and arachnid cell lines existed by the 1990's [Rinkevich 1999]. Although mollusc cell culture has been studied most intensively compared to other marine invertebrates and a 20% increase in the amount of published papers from the period 1988 to 1998 and 1999 to 2004 occurred, no immortalized cell lines could thus far be established [Rinkevich 2005; Travers *et al*. 2008]. Rinkevich postulated that the obstacles encountered during the process of establishing a marine invertebrate cell line are due to the unique requirements of these cultured cells compared to that of vertebrate cell culture. Establishing an overall cell culture protocol for all marine invertebrates is further complicated due the great diversity and unique needs of all the phyla comprising marine invertebrates.

Research has been carried out on several marine mollusc species yielding different degrees of success. Numerous studies have been dedicated to establishing a cell culture line for molluscs and several studies have been carried out on oyster and mussel species,

with several species being examined [Wen *et al*. 1993; Chen and Wen 1999]. Most of these studies experimented with changes in the growth media of the explanted tissue. Different commercially available media were employed with the addition of tissue extracts, fetal bovine serum, hormones, growth factors, egg yolk and fowl serum [Wen *et al*. 1993; Coulon *et al*. 1994; Cornet 1995; Cornet 2000; Barik *et al*. 2004].

Several reports on haliotid cell culture have been published since the 1990's. Recent reports indicate a trend towards using primary cell cultures as target for further studies rather than attempting to create a proliferative cell line (see Table 1.4).

Table 1.4: Recent advances and highlights concerning abalone cell culture.

Species	Cultured tissue	Treatment or procedure	Results	Cells maintained	Reference
H. tuberculata	Haemolymph	Insulin (porcine) and epidermal growth factors (human)	Viability of cell remained constant for at least 6 days. Increased DNA synthesis, but no cell proliferation.	6 days	1
H. discus hannai	Digestive gland	Nine different culture media, Fetal bovine serum (FBS) and salts	Best growth found in ERDF media containing no FBS with high salt concentrations (NaCl, KCl, $MgCl_2$, $MgSO_4$, $CaCl_2$).	5 days	2
H. tuberculata	Mantle	Shell extracts of *Pinctada maxima* added to culture media	Increased cell density during early culture with addition of shell extracts. Cell death correlated with addition of shell extracts at higher concentrations. Increase in enzyme responsible for cell formation.	28 days	3
H. varia	Mantle	Culture media 199 with FBS	Cell growth and proliferation. *In vitro* calcium carbonate crystals formation.	102 days	4
H. tuberculata	Mantle and haemocyte	Calcitonin-related molecules (human) No growth factors (haemocytes)	Low levels of proliferation observed in mantle cells. High metabolic activity for mantle and haemocyte cultures. Calcitonin-related molecules (human) increase the activity of carbonic anhydrase. Calcitonin-related molecules (human) modulates	14 days	5

			activity of target cells in mantle and haemolymph in the process of shell biomineralization.		
H. varia	Mantle	Culture media (L-15, F12 and M199) and tissue extracts	L-15 media prompted better cell yield. M199 yielded better cell adherence. Mantle extract enhanced cell yield. Whole body extract facilitated better cell adherence.	8 days	6
H. tuberculata	Haemolymph	Morphological, cytometric and functional characterization of haemocytes	Two cells types could be distinguished; the abundant large hyalinocytes and less abundant smaller blast-like cells. In some cases another cell type could be distinguished, a basophilic granulocyte.	N/A	7
H. midae	Larval and haemocytes	Investigation into the suitability of primary cultures to serve as subject for future studies	Haemocytes were confirmed to be useful in future studies.	10 to 21 days	8

1: Lebel *et al*. (1996); 2: Kusumoto *et al*. (1997); 3: Sud *et al*. (2001); 4: Suja and Dharmaraj (2005); 5: Auzoux-Bordenave *et al*. (2007); 6: Suja *et al*. (2007); 7: Travers *et al*. (2008); 8: Van der Merwe *et al*. (2010)

1.3.1. Primary mollusc cell cultures

Although primary cell cultures are not immortalized, mantle and haemocytes have been found to be relatively successful in producing primary cell cultures with relative ease and have successfully been used in several studies [Sud *et al*. 2001; Suja and Dharmaraj 2005; Cornet 2007; Van der Merwe *et al*. 2010]. Mantle cell culture has been employed to study the nacre forming properties of abalone tissue that are important in the valuable pearl forming process [Suja and Dharmaraj 2005; Auzoux-Bordenave 2007]. Haemolymph cell culture presents researchers with the opportunity to study the immune response of haemocytes, the main immune effector cells, in an attempt to elucidate the functioning of the immune response of abalone and the effect certain pathogens might have on it. Cornet (2007) evaluated the possibility and potential usefulness of primary mussel mantle cell cultures for the detection of seawater pollutants and the assessment of genotoxic effects these pollutants cause. Cultures were treated with a known pollutant, cadmium, and DNA damage tracked though sister chromatid exchange. Even at the lowest concentration of cadmium, DNA responses were detected. Cornet (2007) concluded that the use of primary cell cultures is feasible for the determination of genotoxicity in seawater samples. A similar study was conducted by Auzoux *et al.* (1993), where primary cultures of bivalve gill were used for pathology studies.

Abalone tissue culture offers several biotechnology opportunities such as the potential for the production of seaweed digesting enzymes as reported by Kusumoto *et al*. (1997). Their study cultured abalone digestive gland cells for the production of enzymes to be utilised in the digestion of seaweed in an economically valuable process. Abalone cell culture also offers the possibility to study novel biochemical pathways that could hold economical advantages such as the pearl forming or shell forming pathways as studied by Sud *et al*. (2001), Suja and Dharmaraj (2005) and Auzoux-Bordenave *et al*. (2007).

1.3.2. Haemocytes

Haemocytes are found in the haemolymph that circulates through the abalone and other molluscs' tissues. Although Travers *et al*. (2008) mentions the extraction of haemolymph by hypodermic needle from the cephalic artery of the animal, haemolymph is generally bled from an incision in the foot of animals sacrificed for this purpose [Auzoux-Bordenave *et al*. 2007]. Although little is known about the invertebrates' immune system, it is well known that

invertebrates do not possess an acquired immune system like that of vertebrates [Roch 1999]. Haemocytes are the main immune effector cell of the invertebrate immune system and although the hematopoietic tissue has not been identified, it is known that the haemocytes are responsible for chemotaxis, lectin-mediated pathogen recognition, phagocytosis and the production of antimicrobial peptides. Haemocytes, however, also play a role in digestion, metabolite transport, biomineralization for shell formation and the repair of injuries to flesh and shell [Auzoux-Bordenave *et al*. 2007; Travers *et al*. 2008].

Travers *et al*. (2008) published the latest and most conclusive characterization of the cell constituents of haemolymph. Due to the uncertainty about the classification of mollusc haemocytes, their study set out to comprehensively classify these cells by the use of several techniques that included light microscopy, cell staining, phagocytosis assays, transmission electron microscopy and flow cytometry analysis. Two cells types were distinguished; the abundant large hyalinocytes with a low nucleus to cytoplasm ratio and the less abundant smaller blast-like cells with a high nucleus to cytoplasm ratio. In some rare instances a third cell type could also be distinguished; a basophilic granulocyte. Both these more abundant cell types are considered to be undifferentiated and immature due to their morphological characteristics. Their study also found thin pseudopodia emanating from haemocytes after adhesion, with adhesion taking place rapidly. Cells were also reported to be able to migrate. These characteristics support the haemocyte's capabilities of chemotaxis for phagocytosis of foreign bodies as well as immune surveillance. Travers *et al*. (2008) concluded by noting that haemocytes of gastropods clearly differ from that of bivalves and that definitions of haemocytes cannot be carried over from bivalves to gastropods. They also noted that presently technology is lacking in completely characterizing the gastropod immune system and haemocytes.

1.3.3. Mantle

Mantle cells are considered to be the layer of cells in direct contact with the inner shell surface. Cultures of these cells are produced by excision of the tissue layer followed by the slicing of the tissue into strips to produce explants that are placed on culture dishes containing mantle culture medium. Primary culture cells emanate from these explants [Auzoux-Bordenave *et al*. 2007]. Primary mantle cell cultures contain a heterogeneous

population of cell types; these include epithelial cells, fibroblast-like cells and glandular mucous cells [Auzoux-Bordenave *et al*. 2007]. Unlike haemocytes, mantle cells exhibit the ability to markedly increase their cell density while maintaining metabolic activity for relatively long periods of time compared to haemocytes.

Mantle cells are responsible for shell formation by transport of calcium and bicarbonate ions through the mantle via the haemolymph and the biomineralization of these compounds to form calcium carbonate bonds that are the building blocks of the shell. The mantle is also involved in the secretion of an organic matrix that facilitates the biomineralization process by interacting with the calcium and bicarbonate ions. This process takes place within the extrapallial space, which is located between the inner surface of the shell and the outer mantle epithelium [Auzoux-Bordenave *et al*. 2007].

1.4. Abalone in South Africa

Six *Haliotis* species namely *H*. *parva* L., *H*. *spadicea* (Donovan), *H*. *queketti* (Smith), *H. alfredensis* (Bartsch), *H*. *pustulata* (Reeve) and *H*. *midae* are found around the southern African coast [Sales and Britz 2001]. Because of its size, only one - *H*. *midae,* known as perlemoen by locals*,* is of economic importance. *Haliotis midae* is therefore the target of harvesting and commercial farming [Genade *et al*. 1988].

1.4.1. Haliotis midae

Natural abalone populations around the South African coast spawn twice a year. This bi-annual spawning is most probably related to seasonal temperature changes that induce sexually mature individuals to spawn [Newman 1967]. Although reports on the spawning of natural abalone populations existed since at least 1967 [Newman 1967], the first report of the successful controlled breeding of *H. midae* was only published in 1988 [Genade *et al*. 1988]. The report described the harvesting of mature abalone from wild populations and the induction of these individuals to spawn. Ova were exposed to sperm for 15 minutes before a wash step was undertaken to remove excess sperm. Hatchlings were observed 14 hours post-fertilization, trochophores after 22 hours, and early veligers at 24 hours, mid-formed cephalic tentacles at 86 hours and settling was achieved at 5 days post-fertilization. Trochophore larvae were measured at approximately 164µm by 190µm, while veligers

measured 207µm by 265µm. It was found that larvae kept at a temperature of 17.5ºC developed slower than larvae kept at 20ºC. The larval stage for animals kept at 17.5ºC lasted 7 days while animals kept at 20ºC completed the larval stage within only 5 days. Genade *et al*. (1988) concluded that larval features and trochophore and veliger behaviour did not differ significantly from descriptions of other haliotid species [Courtois de Vicose *et al*. 2007]. Tarr (1995) found *H*. *midae* to reach sexual maturity at approximately 7.2 years in colder waters, while reports indicate that animals that reside in the warmer waters of the east coast reach sexual maturity at 3 years with the onset of spawning.

South African commercial abalone farming produces up to 934 tons of abalone per year with the production value exceeding ZAR268 million [Britz and Lee 2009]. During 2007, prices reached values of up to ZAR650/kg on the black market [CITES 2007]. As a result of diminishing natural abalone resources and the profits to be made, the South African abalone industry has turned into a booming industry consisting of more than 18 abalone farms contributing more than 80% of the monetary value of the total aquaculture sector in South Africa [Britz and Lee 2009]. Not only is the meat exported, but abalone shells are sold as well. Overall, the abalone industry is an important part of the South African economy, drawing foreign currency and creating employment opportunities [Troell *et al*. 2006].

Increased exploitation by recreational divers and illegal poaching has greatly increased the pressure on abalone resources. South African authorities have had to implement several strategies to try and curb the exploitation and resulting destruction of this resource. In 1953 a minimum size limit of 10.2cm was introduced for abalone harvesting. The following year the size limit was increased to 11.4cm. In 1969 a limit on the maximum catch was instituted for abalone factories. Closed seasons for abalone harvesting was implemented in 1985 with a total allowable catch for specific zones along the South African coast being implemented the next year. With abalone stocks still dwindling, a reduced allowable catch was introduced by 1997. In 2000 it was estimated that illegal catches comprised almost half of the total allowable catch and that 55% of illegal catches were under the size limit [Dichmont *et al*. 2000]. Concerns for the survival of the species ultimately led to the closure of abalone harvest from February 2008 to July 2010 [CITES 2010].

1.4.2. Haliotis midae genetic studies

Harvesting and commercial farming have encouraged the implementation of genetic studies on *H. midae*. Harvesting has led to the need for genetic studies on wild populations that centre on the assessment of overexploitation and characterization of the population for management purposes by assessing the genetic diversity and genetic structure of the population and gathering of information to assist in applying harvesting laws [Roodt-Wilding and Slabbert 2006]. Commercial *H. midae* farming has encouraged the implementation of genetic studies in an attempt to elucidate areas of interest such as genetic diversity and inbreeding of commercial populations. Research has also centred on parentage assignment, marker-assisted selection, reintroduction of commercial population into the wild and harvesting's effect on the wild population size and genetic diversity [Roodt-Wilding and Slabbert 2006].

Genetic studies have already yielded noteworthy results that influence the commercial farming of *H. midae*. A high degree of inbreeding has been observed in other commercial species of abalone (*H. discus hannai*) [Hara and Sekino 2006] and has been confirmed for commercial *H. midae* stock as well [Evans *et al*. 2004; Slabbert *et al.* 2009]. This is most-likely due to the limited founder population resulting in a population bottleneck and genetic drift. A significant variation between the population from the East and West Coast of South Africa has also been indicated in genetic studies, making it a risky practice to outcross possibly divergent populations and perform restocking operations which could affect the fitness of the species for local adaptations [Bester-Van der Merwe 2009]. Several molecular markers have been identified in the *H. midae* genome with AFLP (amplified fragment length polymorphism) markers [Badenhorst 2008] and microsatellite markers [Hepple 2010] having been incorporated into linkage maps. Recently identified SNP (single nucleotide polymorphism) markers [Bester-Van der Merwe 2009; Rhode *et al.* 2010] will increase the density of *H. midae* linkage maps and combined with QTL studies could eventually lead to marker-assisted selection [Slabbert 2010].

In the commercial environment selective breeding has led to great improvement in some species and is the most practical choice in improvement of stock in the commercial environment [Hulata 2001; Hayes *et al.* 2007]. Selective breeding requires the parentage of

offspring to be known and the assessment of target traits [Hayes *et al.* 2007]. Due to the nature of commercial abalone farming as well as abalone biology it is often difficult to determine the parentage of offspring and assess target traits [Rasmussen and Morrissey 2007]. Due to the fact that abalone are broadcast spawners with fertilization of the millions of spawned gametes taking place externally, parentage is often difficult to assess. Abalone's long generation time and slow growth (*H*. *midae* takes four to five years to mature to a commercially suitable size of 100mm [Macey and Coyne 2004]) also further complicates selective breeding by resulting in progress being very slow [Rasmussen and Morrissey 2007].

Gene transfer has therefore become a plausible option for use in the abalone industry. Genes that code for certain favourable traits can be incorporated into the genome of the transformed animal to yield economically beneficial animals that exhibit traits such as faster growth, disease resistance and increased fertility depending on the incorporated gene [Levy *et al*. 2000; Rasmussen and Morrissey 2007].

1.5. Genetic modification

Advances in gene knowledge and manipulation techniques in recent years have opened the door for artificial manipulation of the genome of living cells and animals [Rasmussen and Morrissey 2007]. Since the 1980's several transgenic species of livestock have been produced with techniques progressing from microinjection to advanced techniques that are currently employed to create transgenic cell lines and animals [Robl *et al*. 2007].

Gene transfer techniques can be divided into DNA transfer by biological vectors, such as viruses, chemical and physical methods [Mitrovic 2003]. Each of these types of gene transfer possesses specific advantages and disadvantages. Retroviral vectors can be introduced into ova or embryos at various stages with only a single copy being integrated into the host genome. Drawbacks of this technique include the small size of constructs that can be transferred and the fact that animals produced in this way are generally mosaic with sporadic transfer of the transgene to offspring [Dyck *et al*. 2003]. Viral gene transfection also raises certain biosafety issues depending on the virus used [Mitrovic 2003]. Although viral gene transfer surpasses chemical and physical methods as far as efficiency and stable transfection are concerned, a shift towards using chemical and physical methods has begun due to their

non-infectious nature, low immunogenicity, low cytotoxicity and the possibility of carrying out transfections on a large scale with relative ease [Mitrovic 2003].

1.5.1. Methods of gene transfer

Physical gene transfer methods include electroporation, the application of an electrical pulse to produce pores in the cell membrane through which foreign DNA enters the cell; and biolistic particle bombardment, where heavy metal particles are coated with foreign DNA and propelled into cells [Mitrovic 2003].

Chemical gene transfer involves the use of a transfection reagent that facilitates the uptake of foreign DNA into cells. Lipofection, the use of a cationic lipid as transfection reagent as well as other cationic polymers such as polyethylenimine, has become the most-popular chemical transfection reagents [Iverson *et al*. 2005]. Lipofection reagents facilitate the uptake of DNA by incorporating the DNA into a liposome which binds to the cell membrane to release the DNA molecules within the cell. Polyplex-mediation employs the positive charge of the transfection reagent to bind DNA and transport it over the cell membrane by electrostatic interactions [Mitrovic 2003].

1.5.2. Polyethylenimine (PEI)

Boussif *et al*. (1995) was enticed to investigate polyethylenimine (PEI) as a possible transfection reagent after observing that several polycations, such as polyamidoamine cascade polymers and lipopolyamines, exhibit substantial buffering capacity below physiological pH and are efficient transfection agents. PEI was found to be an efficient transfection reagent for use in many different cell types for *in vitro* and *in vivo* use. PEI also exhibited low cytotoxicity and decreased degradation of inserted DNA, making it a proficient transfection reagent.

PEI is an organic polymer of which every third atom is a protonable amino nitrogen atom, making PEI the organic macromolecule with the highest cationic-charge-density potential. These properties allow PEI to act as a ‘proton sponge’ with substantial buffering capacity at almost all pH levels and makes it possible for PEI to associate with negatively charged DNA, to form a polyplex, and transport this DNA over the cell-membrane for release within the

cytoplasm [Boussif *et al*. 1995]. Since then PEI has become a widely used transfection reagent for delivery of DNA, siRNA, ribozymes and oligonucleotides [Richards Grayson *et al*. 2006; Huh *et al*. 2007].

It was hypothesized that endosome buffering and subsequent DNA degradation protection by PEI may explain the efficiency of PEI as a transfection reagent [Boussif *et al*. 1995]. Kichler *et al*. (2001) investigated the mechanism of polyethylenimine-mediated gene delivery by use of proton pump inhibitors, preventing proton influx and therefore acidification of the endosome. It was demonstrated that the functioning of PEI is dependent on the acidification of the endocytic vesicle and that the transgene could rapidly escape from the endosome once in the cytosol. The positively charged amine groups found in PEI form a complex with the negatively charged phosphate groups of nucleic acids to produce a neutral or slightly positive complex. This complex is stable enough to allow for entry into the cell by crossing of the cell membrane and release of the nucleic acid intracellularly [Richards Grayson *et al*. 2006]. The fundamental steps in chemical gene transfer is the casing of DNA into compact particles, movement of these particles over the cell membrane, the release of DNA into the cytosol, transport of these DNA molecules into the nucleus and the expression of the transported genes [Kichler *et al*. 2001; Von Gersdorff *et al*. 2006].

1.5.3. Gene transfer to abalone

The first gene transfer to abalone was accomplished by Powers *et al*. (1995). Electroporation was employed to introduce a linearized plasmid containing a *Drosophila* beta-actin promoter with a beta-galactosidase gene to fertilized eggs of *H*. *rufescens.* The transgenes were found to be retained in 72% of juveniles for 3 to 7 months. Five other studies have reported on the successfully transfection of abalone: *Haliotis iris* [Sin *et al*. 1995], *H*. *asinina* [Counihan *et al*. 1997], *H*. *diversicolor supertexta* [Tsai *et al*. 1997; Chen *et al*. 2006] and *H*. *discus hannai* [Wang *et al*. 2004]. Tsai *et al*. (1997) and Wang *et al*. (2004) reported the retention of the transgene in 65% and 13.9% of larvae respectively. All studies indicated transfected larvae to have a significantly lower survival rate.

1.5.4. Transgenes

Abalone is a commercial species and transgenesis is foremost for commercial gain. It is therefore of utmost importance that the construct being used in transfection be acceptable to the consumers. A construct containing only abalone genes would be preferable and most probably more efficient in transfection studies than a heterologous construct containing genes from other species. Attempts have been made to elucidate genes that could be responsible for an increased growth rate in abalone [Van der Merwe 2010]. Such genes could possibly be incorporated into a homologous abalone gene construct. Gomez-Chiarri *et al*. (1999) reported on the creation of a gene construct containing the abalone actin promoter. The actin promoter gene is expressed stably in the majority of tissue under most circumstances and would therefore be an ideal promoter for stable expression of possible growth transgenes. By combining constitutive abalone promoters and growth genes, constructs could be created that increases the growth rate of transfected individuals [Gomez-Chiarri *et al*. 1999].

Addition of growth hormone to livestock has been shown to promote bone growth, lipid and carbohydrate metabolism and steroid metabolism and increase production of livestock [Lichanska and Waters 2007]. The use of growth hormone for the enhancement of growth in fish has been examined extensively, with several transgenic species being studied with increased growth, feed conversion and increased protein content of carcasses being observed [Du *et al*. 1992; Chatakondi *et al*. 1995; Nam *et al.* 2001; Lu *et al.* 2002]. It would be expected that addition of a stably expression growth gene would have the same effect on abalone and abalone production. However, little is known about growth hormone genes and the effect administration and transfection with growth hormone would have on abalone growth [Moriyama and Kawauchi 2004]. Molluscs possess growth hormone-like molecules as shown by Lubet (1971), while studying the gastropod *Crepidula fornicate*. A molluscan insulin-like peptide (MIP) has been isolated and sequenced and the series of genes encoding MIP has been identified in *Lymnea stagnalis* [Smit *et al*. 1992]. Growth hormone-like substances have also been isolated from *Haliotis discus hannai* by Moriyama *et al*. (1989), but no protein, homologous to that of the vertebrate growth hormone, has been identified in molluscs.

Trials to investigate the plausibility of vertebrate growth hormone enhancing growth in abalone have been carried out. Morse (1984) immersed *H. rufescens* juveniles in mammalian

growth hormone and insulin solutions and observed enhanced growth of the treated groups. Kawauchi and Moriyama (1991) also observed enhanced growth when treating *H*. *discus hannai* with a recombinant salmonid growth hormone. Trials have also been carried out on other mollusc species, such as the eastern oyster (*Crassostrea virginica*), where recombinant trout growth hormone was used to enhance growth [Paynter and Chen 1991]. The aforementioned trials were all carried out on juvenile animals. Taylor *et al*. (1996) made use of adult *H. kamtschatkana* animals that received intramuscular injections of recombinant bovine growth hormone, recombinant porcine growth hormone, somatostatin or bovine serum albumin. These animals did not exhibit any significant increase in growth. It is therefore clear that growth hormone would only beneficially if applied during the juvenile growth phase.

Insertion of a homologous gene that increases the growth rate of abalone would be ideal for the purpose of creating an abalone with an increased growth rate resulting in improved commercial production. There are however several stumbling blocks that impede the creation of a transgenic *H. midae* expressing a homologous transgene for increased growth: there is no established protocol for the transfection of *H. midae,* nor is there a homologous construct available. Most importantly, the mechanism responsible for growth in abalone has not been identified nor has genes possibly involved in the growth been confirmed.

Recently, genes have been identified that have been linked to differential growth rate in *H. midae* [Van der Merwe 2010]. By confirming the differential expression of these genes within different *H. midae* growth stages that are characteristic for an increased growth rate, it would be possible to assess, to a certain degree, their usefulness as transgenes. Quantitative real-time PCR (qRT-PCR) has emerged as the most-suitable and accurate technique for assessment of differential gene expression [Bustin *et al*. 2009; Derveaux *et al*. 2010] and would therefore be an obvious choice to quantify the expression of putative growth genes in abalone.

1.6. Differential expression analysis

The quantitative real-time PCR technique has proven to be especially suitable for comparative studies to measure the expression of a target gene in comparison with a reference gene also expressed in the sample [De Gregoris *et al*. 2009]. Two standard

procedures are generally employed; either absolute or relative quantification. Absolute quantification requires suitable standards of accurate and known concentration which allows the starting concentration of samples to be accurately determined. Due to the need to have a reliable set of standards this method is considered to be more expensive and labour-intensive than relative quantification [Pfaffl 2004].

Relative quantification strategies compare and normalise samples to reference genes to eliminate unspecific variation as a result of differences caused by sample preparation, RNA extraction and reverse transcription efficiency. The reference gene should possess stable transcription levels in all samples being tested and should be impartial to experimental treatment. This method may be open to more experimental variability between different runs, days and laboratories because it is not based on a standard [Sellars *et al*. 2007]. The overall conclusion obtained from absolute and relative quantification concerning the expression of genes should however be similar providing that all experimental procedures are correctly implemented and follow the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines [Bustin *et al.* 2009].

During the real-time PCR reaction a fluorescent intercalating doublestranded-DNA binding dye (e.g. SYBR green) emits fluorescence that is recorded by the PCR instrument. The emitted fluorescence is directly related to the number of amplicons generated during the reaction and follows four distinct phases; an exponential phase hidden by background fluorescence, an exponential phase that can be differentiated from background fluorescence, a linear amplification phase and a final plateau phase. All downstream quantification will be carried out on data obtained during the exponential phase. The exponential phase is the only phase where the starting RNA is directly proportional to the amount of product. The amount of starting RNA being the target of quantification studies [Pffafl 2004]. The crossing point value, a value that corresponds to the number of PCR cycles necessary for a sample to reach a defined fluorescent intensity, is used for comparison of samples and references in downstream quantification [Guénin *et al*. 2009].

1.7. Aim of the study

This study therefore endeavoured to create an optimized protocol for the transfection of *H. midae* that could in future by used to incorporate genes associated with increased growth into the genome of commercial abalone. Polyplex-mediation, using the transfection reagent polyethylenimine (PEI), was employed in the transfection process. Quantitative real-time PCR was utilized to confirm differential expression of growth-related genes. Due to the novel nature of this research, several preliminary investigations into autofluorescence, transfection reagent cytotoxicity, and target tissue studies were also carried out to facilitate the successful transgenesis of *H. midae.*

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2. Fluorescence

2.1. Introduction

Fluorescence refers to the property of substances to radiate energy in the form of light of a longer wavelength after being irradiated with light of a shorter wavelength. This emission of light is due to the return of excited molecules to their ground state by the emission of a photon. The initial excitation of molecules is achieved by absorption of photons by a fluorophore from a light source, such as a UV light [Rost 1992; Shapiro 2003].

It has been noted that several organisms have the ability to fluoresce, this being referred to as autofluorescence; thereby separating this natural form, from fluorescence induced by the insertion of a fluorescent molecule or gene. The gene responsible for the autofluorescence of the jellyfish, *Aequorea victoria*, has been isolated and has revolutionized the field of molecular biology and biotechnology. This green fluorescent protein (GFP) produces a fluorophore capable of transmuting blue or ultra-violet luminescence to green fluorescence with an excitation peak at a wavelength of 395nm and an emission peak at 475nm. The GFP gene has proven to be truly versatile and has been utilized in several different *in vivo* and *in vitro* applications, including expression in *Saccharomyces cerevisiae*, *Drosophila* species, *Caenorhabditis elegans*, *Mus musculus* and human tissue cell cultures [Johnson *et al*. 1962; Cubitt *et al.* 1995; March *et al*. 2003].

Improvements to the GFP has yielded an enhanced green fluorescent protein (EGFP) that exhibits excitation and emission maxima at the same wavelengths as that of the wild-type, but with a 40-fold increase in fluorescent yield compared to the wild-type [Crameri *et al*. 1996]. Further studies have also yielded another fluorescent protein, DsRed, from a marine organism. DsRed was isolated by Matz *et al.* (1999) from coral from the *Discosoma* genus and is responsible for the red colouration of this coral. While red-shifted GFP mutants were already available at this time, none of them had an emission maximum longer than 529nm. DsRed represents one of the first isolated fluorescent proteins to have an excitation and emission peak at such long wavelengths, 558 and 583nm respectively [Baird *et al.* 2000]. The field of biotechnology and the study of biology has therefore greatly benefitted from the autofluorescence of aquatic organisms [Shaner *et al*. 2006; Prescott and Salih 2009].

The development of a successful novel transgenesis protocol includes the selection and optimization of a transgenesis technique, creation of an expression vector and the selection of stable transgenic line [Gómez-Chiarri *et al*. 1998]. A reporter gene is essential in the optimization of a gene transfer protocol. Fluorescent, luciferase and GUS reporter systems are the most-widely used reporter systems in transgenesis research. The luciferase and GUS reporter systems require the use of specialized imaging systems and substrates, while the fluorescent reporter gene only requires fluorescent detection by a fluorescent microscope [De Ruijter *et al*. 2003]. Due to fluorescent reporter genes' versatility and proven effectiveness in other organisms and also the relative simplicity of the fluorescent gene reporter system compared with other reporter gene systems, fluorescent genes were the reporter gene of choice for the optimization phase of this project.

Although the autofluorescence encountered in some species has been of great advantage to the molecular biology field, autofluorescence has also been the nemesis of many researchers contending with autofluorescence at the same wavelength as the fluorescent protein of interest [Yentsch and Horan 1989]. It has been noted that several aquatic organisms have the ability to autofluoresce [Yentsch and Horan 1989; Weissleder and Ntziachristos 2003]. Prescott and Salih (2009) reported that several marine organisms such as coral, anemones and zoanthids have fluorescent emissions in the cyan (470nm to 499nm), green (500nm to 520nm) and red (520nm and longer) wavelengths. Mollusk species exhibiting autofluorescence have also been reported; the eastern oyster, *Crassostrea virginica* [Buchanan *et al*. 2001], freshwater pond snail, *Lymnaea stagnalis* [Abe *et al.* 2009] and boring clam, *Pholas dactylus* [Shimomura 2009]. The autofluorescence in the eastern oyster and freshwater pond snail studies were documented only because it had an impact in the observation of inserted GFP expression. Both studies recorded green autofluorescence when viewing tissues under a FITC filter.

Prior to investigating GFP and DsRed as reporter genes for use in preliminary transfection studies in *Haliotis midae*, it was necessary to investigate the fluorescence of different abalone tissues that could serve as possible targets for transfection studies to ensure that autofluorescence would not interfere with the detection of transgene expression. *Haliotis midae* gametes, larvae and haemocytes were fluorescently examined.

2.2. Materials and methods

Haliotis midae gametes were obtained from a commercial abalone farm during routine spawning events. At least three male and female broodstock individuals contributed to the pool of gametes that was examined. Gametes were examined fluorescently within 60 minutes of spawning. Fertilization of ova and settling of spat was carried out by farm personnel as part of the commercial production process. Samples were obtained within an hour post-fertilization and were fluorescently monitored during development and maintained in filtered sea water (FSW) at 18ºC for 247 hours (5 days). A sample of two week old settled spat was also obtained and investigated fluorescently. Some gamete and larval samples were preserved in 100% ethanol for examination at a later stage.

Adult abalone haemolymph was fluorescently examined after the haemolymph was bled from the animals and seeded on well-plates (see Chapter 3: Target tissue, for the complete protocol). All samples (gametes, embryos, larvae, settled spat and haemolymph) were examined using an Olympus IX51 fluorescent microscope with an EGFP (excitation: 450nm to 460nm; emission: 500nm to 550nm) and FITC (excitation: 460nm to 500nm; emission: 510nm to 560nm) filter. Ova and larvae (16-24 hours post-fertilization) were also examined using an Olympus IX81 motorized inverted microscope with multiple filters (Fura, DAPI, CFP, GFP, FITC, YFP, Cy3, Texas-Red, Cy5) covering most of the colour spectrum to determine the range of fluorescence of these tissues.

An EGFP expressing construct, pTracer-CMV2 (Invitrogen) was used to transfect *Escherichia coli* (One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen) and Hep2G (ATCC) cells in order to compare the autofluorescence of abalone with these cell lines. Hep2G cells were also transfected with CMV-DsRed-Express (Clontech) that enables the expression of a red fluorescent protein in eukaryotes. Both cell lines were cultured for 24 hours according to the manufacturer's specifications before chemical transfection.

2.3. Results

A very strong fluorescent signal was observed when examining the fresh and preserved ova, embryos and larvae (see Figure 2.1) using the EGFP and FITC filters. The fluorescent signal observed for the ova, embryos and larvae are of the same magnitude as that of transformed *E. coli* cultures expressing EGFP (see Figure 2.2). Ova and larvae examined at additional

excitation and emission wavelength demonstrated fluorescence at a wide spectrum of excitation and emission wavelength. Although fluorescence was observed at a wide spectrum of excitation, the most intense fluorescent signal was observed in the same emission range as that of CFP (cyan), EGFP, FITC and YFP (yellow) (430nm to 530nm). Ova and larvae (examined within 24 hours post-fertilization) exhibited no or a very low fluorescent signal at excitation at the extremes of the colour spectra. These samples also exhibited only one colour fluorescence. A fluorescent signal was not present or faint when samples were excited with light of very long or short wavelengths. Short wavelengths of 340nm to 380nm and wavelengths longer than 572nm did not excite samples to fluorescence. There was no difference in intensity or spectra of fluorescence between fresh and preserved samples.

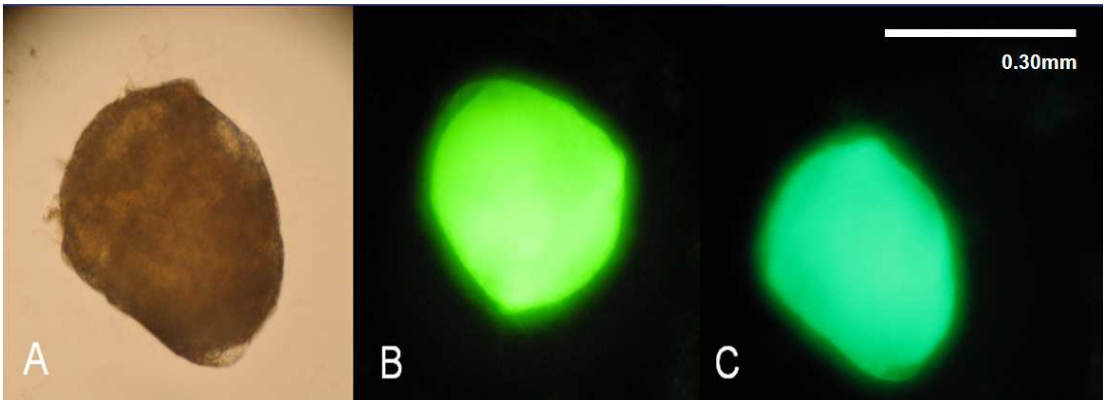

Figure 2.1: A larva of 16 to 18 hours post-fertilization examined with 60 times magnification with a light microscope (A) an EGFP filter (B) and FITC filter (C).

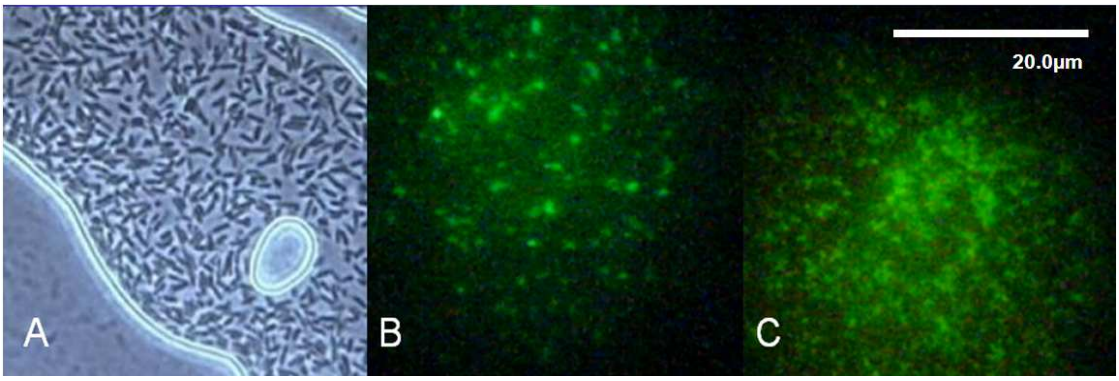

Figure 2.2: Transformed *E. coli* culture expressing the EGFP protein contained in the pTracer-CMV2 gene construct when examined under 100 times magnification with a light microscope (A) and exhibiting a strong fluorescent signal when examined under an EGFP filter (B) and a FITC filter (C).

When larvae were allowed to develop for more than 24 hours post-fertilization, the pattern and colour of the fluorescent emission changed. As larvae developed the green fluorescence observed in the fertilized ova was replaced by yellow-green fluorescence and later by yellow-green as well as red fluorescence (under FITC filter) (see Figure 2.3). This pattern of fluorescent emission remained present in the settled spat as well (see Figure 2.4) The green fluorescence observed under an EGFP filter remained throughout the life stages, but was confined to certain tissues as the larvae developed. It was noticeable that the tissues emitting a red fluorescence under the FITC filter did not emit a green fluorescence when viewed under an EGFP filter.

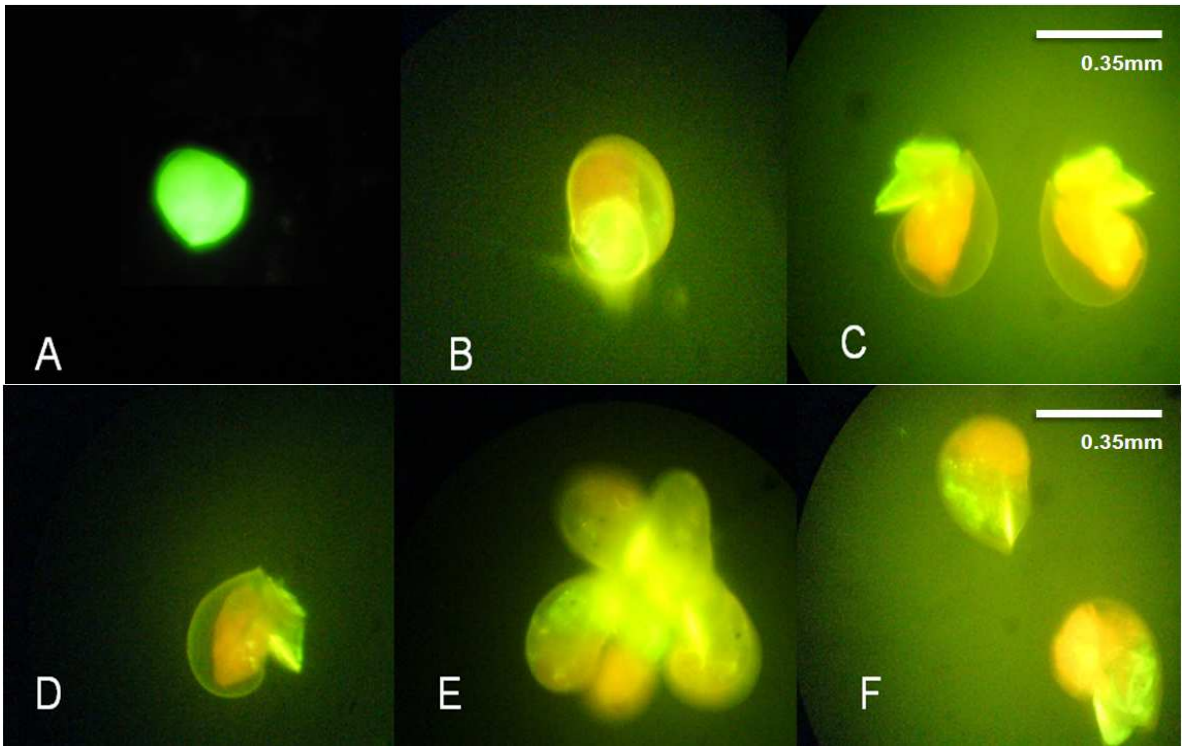

Figure 2.3: Abalone undergo a metamorphosis during larval development with the associated change in expression pattern of developmental proteins. This can be fluorescently observed as the larva's fluorescent emission changes from yellow-green 18 hours post-fertilization (A) to the emission of yellow-green as well as red, 30 hours post-fertilization (B). After 57 hours larvae display a clear distinction between red and yellow-green emitting tissues (C). This pattern of emission is evident in larvae of 79 hours (D) as well as larvae of 127 hours post-fertilization (E) and until at least 5 days

(247 hours) under laboratory conditions without settling cues being introduced to the larvae (F). All observations were done using a FITC filter.

Two week old spat also exhibited autofluorescence of the same magnitude as that of larvae. The spat viewed under a FITC filter exhibited the same localization of yellow and red fluorescence as observed in the larvae, while larvae viewed under an EGFP filter exhibited the same green fluorescence observed in larvae as well (see Figure 2.5). The areas in which the red fluorescence was exhibited in larvae encompassed most of the tissue enveloped by the newly formed shell, while the red fluorescence in the spat was confined to the area surrounding the dorsal ganglia.

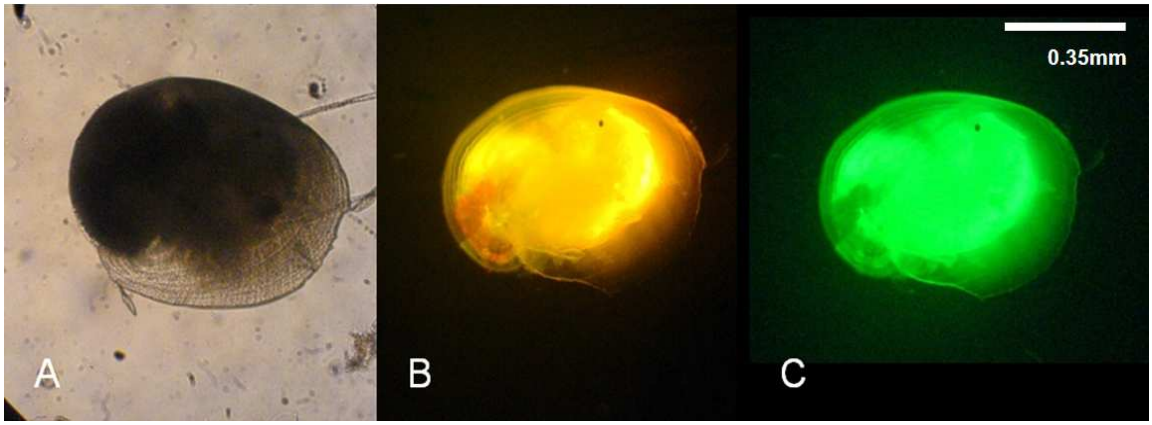

Figure 2.4: A two week old spat (A) (settled according to farm production procedures) exhibited red and yellow fluorescence in specific tissues under a FITC filter (B), while exhibiting green fluorescence when viewed under a EGFP filter (C).

The fluorescence observed in the transfected Hep2G cells were of the same magnitude as the fluorescence exhibited by 24 hour and older larvae (see Figure 2.5). Hep2G cells transfected with EGFP and DsRed exhibited a bright green and red fluorescence when viewed under an EGFP and FITC filter respectively.

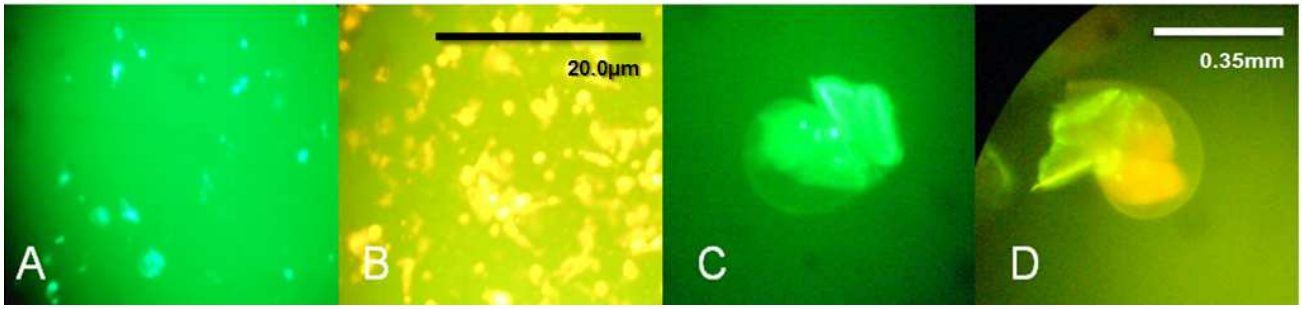

Figure 2.5: Untransformed Hep2G cells did not fluorescence under EGFP or FITC filters. Cells transformed with EGFP (A) and DsRed (B) however fluoresced bright green and red respectively when viewed under EGFP and FITC filters. Untransformed 3 day old larvae exhibited the same emission spectra when viewed under FITC (C) and EGFP (D) filter as that of the transformed Hep2G cells.

Sperm cells and cultured haemocytes did not yield any fluorescent signal at any wavelength tested. It was however observed that haemocytes started to show slight autofluorescence when the cells were examined after several days of culture. This is most probably due to the slow senescence of these cells.

2.4. Discussion

The strongest fluorescent signal was obtained with filter sets for fluorescent proteins in the centre of the light spectra, with faint fluorescence observed with filters at the extremes of the light spectra when examining larvae of less than 24 hours old. The fluorescent signal of *H. midae* ova, embryos and larvae (less than 24 hours old) most probably follows a bell-shaped curve along the light spectra; the highest fluorescent peak at the centre of the spectra in the green range and tapering off to the shorter and longer wavelengths. Altering the excitation wavelength when investigating the fluorescence of a single fluorophore, should not alter the emission wavelength. Since shifting of the excitation wavelength altered the emission wavelength for ova and larvae (less than 24 hours post-fertilization), the presence of more than one fluorophore is assumed [Rost 1992]. This was definitely the case when examining larvae (more than 24 hours post-fertilization) that had developed further tissues that exhibited a red fluorescence together with the yellow-green fluorescence observed in larvae less than 24 hours post-fertilization. The intense green fluorescence was also observed to change to a yellow fluorescence as larval development progressed. This emission pattern remained the

same in the two week-old spat, although the tissues' localities changed due to the ongoing development of the larvae.

Flavinproteins are well known for their fluorescent qualities, usually fluorescing in the yellow-green range (500nm to 600nm) and have been proven to be the main autofluorescent species in an assortment of cell types [Aubin 1979; Benson *et al.* 1979; Van den Berg 2001; Shapiro 2003]. The lecithotrophic larvae of abalone are presumed to contain ample yolk for survival until attachment and feeding. The yolk is provided by the ova. It is therefore assumed that the ova contain highly concentrated proteins and nutrients for this life stage of the larvae [Takami *et al.* 2002]. Seguineau *et al.* (2001) demonstrated that the ova and larvae of the scallop, *Pecten maximus*, contain high concentration of riboflavin which is highly utilized in larval development. This would explain the fact that the autofluorescence is only observed in the ova and larvae and not the sperm cells and changes to a paler yellow as the larvae develops and adapts to a benthic existence.

The biological function of fluorescent proteins has not been elucidated, although several suggestions have been made for their function in other species. These suggestions range from these proteins being involved in predator-prey behaviour to the reduction of photobleaching of light-sensitive organisms [Prescott and Salih 2009]. These suggestions are mainly based on data collected from coral; the function of fluorescent proteins in abalone however remains obscure.

Our results clearly indicate that using merely EGFP as a reporter gene and visual detection of expression in transfection studies in *H. midae* would be futile if fluorescent verification was the only method used to confirm transfection. However, Wang *et al.* (2004) reported making use of the pTracer-CMV2 gene construct containing EGFP as a reporter gene in transfection studies of *H. discus hannai*. Their study used pTracer-CMV2 to transfect sperm and ova and examined the ova end embryos using an Olympus fluorescent microscope (filter set and model not mentioned). No mention was made of any observed autofluorescence and the filter set that was employed in their study could not be confirmed by personal communication. There is no other report of fluorescence in abalone tissue, gametes or larvae that we are aware of. Other mollusc species have however recently been considered for studies similar to the current study and found to be exhibiting green autofluorescence. Buchanan (2001)

observed eastern oyster (*Crassotrea virginica*) larvae to exhibit green autofluorescence during a transfection study utilizing a GFP gene. Abe *et al*. (2009) briefly stated that embryos of their test subject, the freshwater pond snail (*Lymnea stagnalis*) also exhibit "relatively greenish autofluorescence".

Similarly, DsRed would not be ideal for use as a visual fluorescent reporter gene due to the red fluorescence that larvae and spat emit. Selection of transfected individuals expressing the transgene would be impracticable in trying to discern autofluorescence from transgene emissions.

Autofluorescence can be combated by quenching of the fluorescent molecule. Use of these quenching techniques could however prove to be impractical for use in live abalone ova and larvae. Some quenching agents could cause the immediate death or the malformation of the ova and developing larvae [Weber 1950; Van den Berg 2001]. Furthermore, the exterior protective layer of the ova and especially the larvae would prevent substances to enter freely to bind with fluorescent proteins; thereby inhibiting these substances' quenching capabilities [Esponda 2005].

The most convenient way in which to circumvent the problem of autofluorescence would be to make use of a fluorescent protein reporter that fluoresces outside of the spectra observed with ova and embryos; for *H. midae* therefore either at very short or long wavelengths. DsRed has an emission peak at a very long wavelength, but other fluorescent proteins, such as mCherry and mStrawberry have emissions and excitations at even longer wavelengths, [Shaner *et al.* 2005]. These proteins however, also run the risk of being obscured by the autofluorescence exhibited by ova, embryos and larvae or could potentially be indiscernible when compared to the autofluorescence of the larvae and spat.

The use of flow cytometry to distinguish slight variations in intensity of fluorescence between transfected and untransfected gametes would be plausible. Erhardt *et al*. (2006) made use of flow cytometry to determine the efficacy of PEI as transfection reagent for use in different cell lines by subjecting GFP transfected cells to flow cytometry. GFP positive cells were easily distinguished from cells that were not expressing the fluorescent protein. Furlong *et al*. (2001) made use of fluorescence-activated cell sorting to isolate GFP expressing *Drosphila* and

Caenorhabditis elegans embryos from wild-type embryos. A measure of autofluorescence was also observed during their study, although this did not pose a problem for accurate sorting of cells. Buchanan *et al*. (2001) encountered autofluorescence in haemocytes of the eastern oyster, *Crassostrea virginica,* when attempting to execute flow cytometric analysis to determine the expression of red-shifted GFP in transfected cells. Their study therefore found it necessary to record cells as being positive for GFP expression when their fluorescence exceeded a certain threshold that was greater than the autofluorescence.

It is therefore clear that using fluorescent proteins, specifically EGFP and DsRed, as a visual reporter gene in *H. midae* tissues is an impractical task. The current study therefore opted to alternatively employ PCR to verify the presence of the transgenes after transfection treatment. However, the fluorescence of target tissue were still be monitored microscopically and by utilizing flow cytometry analysis.

2.5. References

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3. Target tissue

3.1. Introduction

Transfection studies are concerned with the transfer of exogenous DNA to cells for the expression of this DNA by the cells. This may sound clear-cut however, several factors and variables need to be optimized to establish optimal transfection and stability of transfected lines and/or tissues [Hamm *et al.* 2002; Erhardt 2006]. The specific tissue targeted for transfection studies is one of the factors playing a pivotal role in the success of the study. The type of cells or tissues targeted depends on the desired overall outcome of the study as well as the ability of these tissues to be optimally transfected. Although the desired outcome of transfection studies dictates the overall choice of target tissue, several other parameters need to be factored in when targets are being selected. These parameters include: the ease of obtaining the desired target tissue, stability or ability to maintain or grow the target tissue, transfectablity of the tissue, efficacy of transfection and the long-term expression and transfer of the transgene, and the intended use of the transfected tissue [Niu and Liang 2008].

3.1.1. Abalone target tissue

Numerous tissue types and animal species have been targeted for transfection studies [Niu and Liang 2008]. Several cell lines of different tissue types [Horbinski *et al*. 2001] have also been used for transfection studies and several human, animal and insect cell lines are available commercially for this purpose [Robl *et al*. 2007]. To date, gene transfer to abalone has focused on germline transfection and has therefore focused on sperm [Sin *et al*. 1995; Tsai *et al*. 1997; Wang *et al*. 2004], fertilized ova [Wang *et al*. 2004] and embryos [Powers *et al*. 1995].

Gamete, embryo and gonadal transfection offers possible germline transmission of the transgene and the production of stably transfected individuals with the transgene being expressed in all or several tissue types contained in the individual [Robl *et al*. 2007]. Therefore, if the required outcome of a transfection study is an individual expressing the gene of interest in all or several selected tissues, transfection is usually carried out at the gamete or embryo stages, or on the gonads of the parental generation. This can however still result in chimeric individuals containing a combination of wild-type and transgene-containing cell lines [Esponda 2005; Niu and Liang 2008].

Transfection carried out on cell cultures, originating from differentiated cells, will not directly yield a GM animal and therefore neither germline transmission of the transgene. Transfection studies carried out on cell culture could however contribute valuable information concerning the optimal transfection protocol and contribute to the understanding of the effects transgenes could have on certain tissues. Transfection studies carried out on cell cultures are generally less costly and are less tightly regulated by authorities concerned with the generation of a transgenic animal [Dyck *et al*. 2003]. Furthermore, in the case of a proliferative cell line, factors such as the availability of gametes and fertilization parameters do not have to be taken into account. The possibility of a stably transfected animal line, in the case of gamete and embryo transfection, is therefore juxtaposed against the convenience and experimental opportunities transfection of cell culture offers.

The aim of the current study was the creation of a transgenic abalone containing an inserted plasmid present in all cells of the adult animal and being expressed by the appropriate cells. In an attempt to succeed at this goal, abalone gametes, fertilized ova or embryos needed to be transfected. Spawning of mature abalone, *in vitro* fertilization and maintenance of gametes and fertilized ova and embryos was therefore necessary. Due to the lack of information on the exact fertilization parameters of *H. midae* species and the lack of knowledge of practical techniques in facilitating the effective housing and maintenance of gametes and embryos, these parameters needed to be elucidated before further investigations could be carried out.

As an additional goal of the study, abalone cell culture was chosen as target tissue for transfection. Due to the fact that the creation of an abalone and invertebrate proliferative cell line has been unsuccessful worldwide [Rinkevich 2005; Travers *et al*. 2008], primary abalone cell cultures were used in this study to assess their usefulness as transfection target. Although recent literature concerning abalone cell culture has centred on haemocyte and mantle tissue culture [Lebel *et al*. 1996; Azoux-Bordenave *et al*. 2007; Travers *et al*. 2008], culture of abductor muscle tissue and epipodocyte tissue was also undertaken.

3.2. Materials and methods

3.2.1. Abalone gametes

Sperm, ova and embryos were obtained from abalone farms at Gansbaai or Hermanus. Broodstock were induced to spawn by addition of measured quantities of hydrogen peroxide to broodstock tanks after which fertilization was carried out by farm personnel and embryos siphoned into containers for settling. After embryos had settled sufficiently (within 10 minutes of settling) they were pipetted from the bottom of the containers with vacuum pipettes into glassware.

When unfertilized ova were required the same process of settling and siphoning was repeated, but prior to sperm addition. After collection, ova were left to settle in the glassware and concentrated. Sperm was taken directly from upper water columns in the housing tanks of male animals. Gametes and embryos were housed in either glass bottles or 50ml Greiner Falcon tubes until experiments were carried out. All samples were a mixture of gametes or embryos from at least 5 broodstock animals.

An inverted light microscope (Olympus IX51) was employed to view and count sperm and ova to determine their concentration. A haemocytometer was used to ensure an accurate sperm count. When necessary, sperm was concentrated by centrifugation at 3500g for at least 1 minute.

3.2.2. Fertilization parameters

Initially, fertilization and transfection experiments were carried out in well-plates (24-well, 12-well and 6-well) containing 2ml to 8ml of filtered sea water (FSW). The limited volume of water however restricted the scope of the trial and therefore further fertilization experiments were conducted in larger volumes. Fertilization ratio experiments were henceforth carried out using 250ml Erlenmeyer flasks containing FSW. The volume of water necessary to maintain optimum conditions for fertilization was determined by making use of a series of flasks containing different volumes (50ml, 100ml, 150ml, 200ml) of FSW. Fertilizations were carried out in the different volumes of water and the efficiency of fertilization was determined by a larval count. To determine the optimal fertilization ratio of sperm to ova; $1.0x10^3$/ml ova was exposed to a series of sperm concentrations ($5x10^1$; $5x10^2$; $2.5x10^3$; $5x10^3$; $5x10^4$) in 200ml

FSW. The viability period of sperm and ova was examined by conducting fertilizations at time intervals of 30 minutes starting immediately after spawning and continuing until 240 minutes (4 hours) after spawning. Larvae were counted after at least 16 hours post-fertilization. Samples of 100µl were taken from Erlenmeyer flask and the number of normally hatched larvae as well as abnormal larvae was documented to determine the percentage of normally hatched larvae in each sample. All samples were incubated at 18ºC. All experiments were carried out in triplicate (see Roux 2011 for a comprehensive *H. midae* reproductive investigation as part of a Ph.D. study).

3.2.3. Tissue culture

Attempts were made to culture several differentiated tissues types; haemolymph, mantle, abductor muscle and epipodocyte tissue. The tissues were aseptically harvested and cultured according to an adapted protocol taken from Auzoux-Bordenave *et al*. (2007) and from information obtained from Mathilde van der Merwe through personal communication (see Van der Merwe *et al*. 2010]. A 70% (v/v) ethanol solution was used to gently remove excess mucous from experimental animals (at least 4cm to 6cm in length). Tissue was harvested as aseptically as possible in a laminar flow cabinet by using a sterile scalpel to remove the mantle, slice off thin sections of abductor muscle and remove the epipodocytes (see below for protocol relating to the collection and culture of haemolymph). Tissues were transferred to a Petri dish and covered with an antibiotic wash solution (see Appendix for constituents) for 90 minutes. The tissues were subsequently transferred to a 15ml tube to which enough antibiotic wash solution was added to cover the sample. Samples were incubated overnight at 4℃. The antibiotic wash solution was replaced the next day and the samples were incubated for another 24 hours at 4℃. Samples were transferred to a Petri dish that contained antibiotic wash medium and culture medium (1:1) (see Appendix for constituents) that covered samples while they were cut into 1mm^2 pieces using a sterile scalpel. Tissue samples were thereafter placed in a 6-well culture plate (Cellstar, Greiner Bio-one) that had been coated with culture medium and incubated 30 minutes at room temperature for explants to adhere to the culture plate. Culture medium (1ml) was gently added to each well to avoid dislodging of explants. Culture dishes containing the explants were cultured at 18℃ in a Hotpack low temperature incubator and monitored for growth. This procedure was also carried out excluding the

second overnight antibiotic incubation at 4℃ as we ll as the first overnight antibiotic incubation in the case of the abductor muscle tissue.

3.2.4. Haemocytes

A 70% (v/v) ethanol solution was used to gently remove excess mucous from live experimental animals (at least 4cm to 6cm in length). Under aseptic conditions the base of the animal's foot was stretched away from its shell to expose the abductor muscle. A sterile scalpel was used to remove the foot from the visceral mass by severing the abductor muscle. Care was taken not to pierce the viscera. The severed foot muscle was placed in a Petri dish to bleed out. The use of a hypodermic needle to extract haemolymph from the cephalic artery of large (more than 10cm in length) individuals was also investigated because this method of haemolymph collection would not require the sacrifice of a live animal. After collection an equal volume of antibiotic wash solution was added to the haemolymph. This mixture was filtered through a 70µm cell strainer into a sterile polypropylene tube. An equal volume of Alsever solution (see Appendix for constituents) was added to the antibiotic wash solution/haemolymph mixture. Samples were placed on a vortex for at least 1 minute to eliminate clumping of cells in the solution. The concentration of the cells in the solution was determined using a haemocytometer. Samples were seeded at a density of 5.0 to 8.0x10^4 in a 96-well plate (Cellstar, Greiner Bio-one) and incubated at 18℃ in a sterile incubator for 90 minutes for adhesion to take place. The supernatant was removed and carefully replaced with a solution containing 50% (v/v) of the antibiotic wash solution and 50% (v/v) of the culture medium (100µl/well) and incubated for 90 minutes before the medium was removed and replaced with culture medium (100µl/well). Culture plates were incubated at 18℃ in a Hotpack low temperature incubator until further experiments were carried out.

3.3. Results

3.3.1. Fertilization parameters

To facilitate optimum fertilization in an Erlenmeyer flask, a volume of 200ml FSW was necessary. The sperm to ova ratio that yielded the best fertilization efficiency was determined to be 1:5000 with the sperm concentration being 2.5x10^5 sperm/ml. Gametes spawned less than 2 hours prior to fertilization yielded the best fertilization results with gametes that were spawned more that 2 hour prior to fertilization, showing a marked decrease in fertilization

success. Optimal results were therefore obtained with gametes that were spawned less than 2 hours prior to fertilization, with fertilization being carried out in a 250ml Erlenmeyer containing 200ml FSW with $1.0x10^3$/ml ova and $5x10^7$ sperm (see Roux 2011 for more information).

Larvae could only be housed less than 7 days in the laboratory environment. The large volumes of aerated FSW and settling cues, amongst other things, necessary to meet the needs of the developing larvae could not be met in the laboratory environment.

3.3.2.1. Tissue culture

The culture of the mantle, abductor muscle and epipodocyte tissue was inefficient for the overall aim of transfection. The cells from these tissues did not exhibit sufficient growth and adhesion necessary for the rigors of transfection. Although growth and a high cell yield was expected from mantle tissues, there were few, if any, cells emanating from mantle tissue explants (see Figure 3.1). Several attempts were made to culture these cells which all yielded the same disappointing results. Modifications were made to the culturing protocol whereby the second overnight antibiotic incubation at 4℃ as we ll as the first overnight antibiotic incubation in the case of the abductor muscle tissue was excluded from the protocol to ease the strain the cells endured during the antibiotic washes. These modifications did not yield any improvement in culture. The contamination of the reagents or media, plasticware and the possibility of faulty laboratory equipment was excluded as the cause of the unsuccessful culture of these tissues due the fact that that all reagents, media and laboratory equipment used in these cultures were also used in the successful culture of the haemocytes.

The relative ease of the culture of the haemolymph cells made these cells the obvious choice for further experimentation. The haemolymph cell culture exhibited numerous attached living cells (see Figure 3.1) and remained viable for at least 7 days before cells started to lose their turgid round structure and detached from the culture plate. After attachment, the haemocytes did, however, not proliferate to cover the culture plate to confluency or increase the number of cells.

The antibiotic regime used for the other tissue types was sufficient to prevent contamination of these relatively non-sterile tissue types. The haemolymph cell cultures also remained predominantly uncontaminated even when not including Amphotericin B (fungicide) in the medium composition.

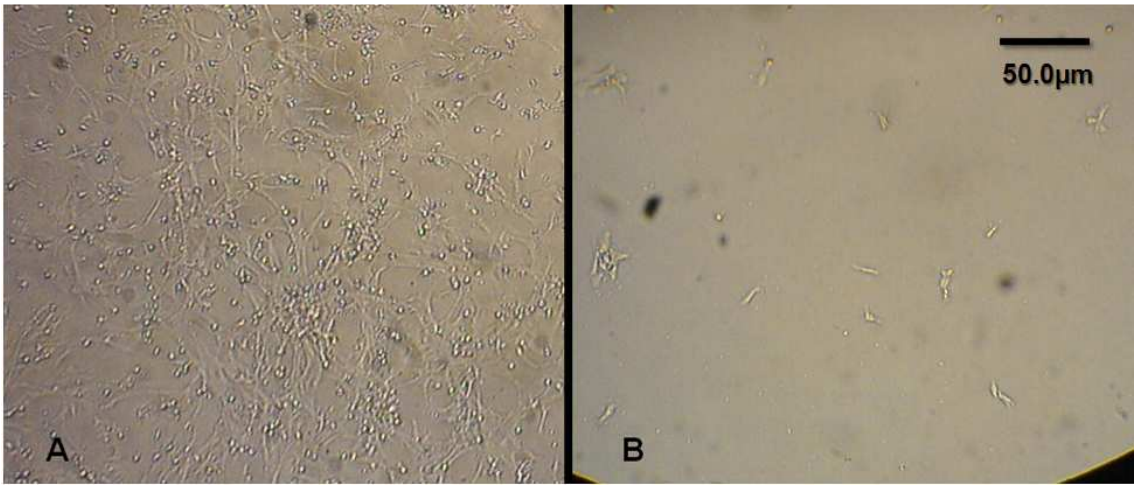

Figure 3.1: One day old haemocyte cultures seeded at 5.0 to 8.0x10^{4}cells per well in a 96-well plate exhibited a high density of attached cells and the presence of pseudopodia soon after seeding. Cellular network visibly extend between cells (A). Mantle cell culture yielded a meagre number of cells that did not seem to attach to culture plates, even after 3 days of growth (B).

3.3.2.2. Haemolymph

Sufficient amounts of haemolymph were obtained to seed one or two 96-well plates by bleeding out an animal of at least 6.0cm in length. These animals needed to be housed at 18ºC in well aerated seawater and undergo a minimum amount of stress to yield adequate amounts of haemolymph suitable for cell culture. Extraction of haemolymph from the cephalic artery of large (more than 10cm in length) animals did not deliver sufficient amounts of haemolymph for culture without causing the death of the animal. Numerous large animals would therefore be necessary to collect enough haemolymph for a single culture experiment. Due to the difficulty in obtaining such animals and the possible health risks caused to the animal by this practice, it was decided not to continue with it.

The haemocytes exhibited the characteristic formation of pseudopodia within an hour of placing then in culture wells. The pseudopodia continued elongating for several hours after adherence and frequently formed networks with groups of other pseudopodia in its proximity. Although it was difficult to distinguish the several cell types Travers *et al*. (2008) described, epithelial/amoeboid cells and fibroblast-like cells could easily be identified (see Figure 3.2). The amount of fibroblast-like cells increased from the time of seeding for a period of 24 hours. Thereafter, a slow decline in these cells was observed coupled with withering of the rounded epithelial/amoeboid cells until cells started to detach from the culture wells after more than a week of culture.

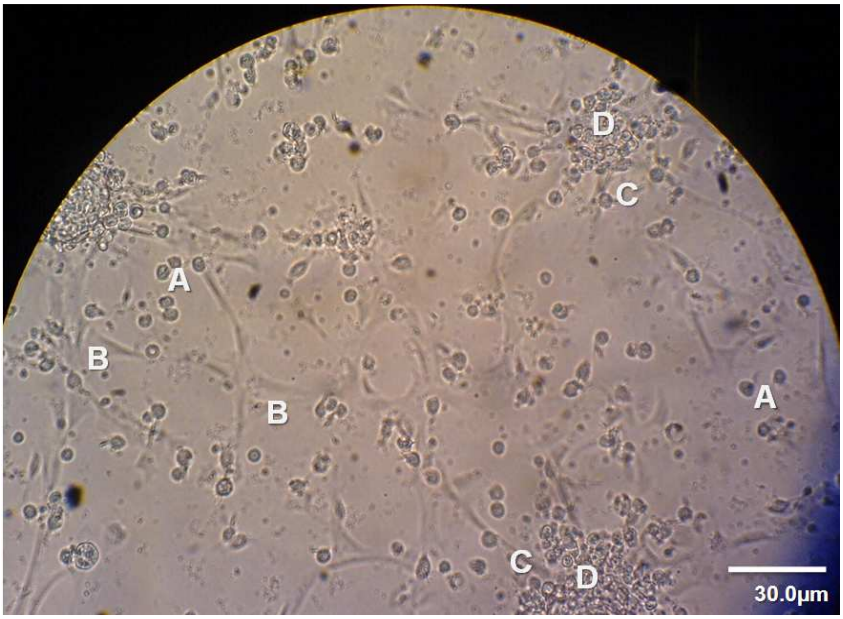

Figure 3.2: An one day old primary haemocyte cell culture exhibiting characteristic cell types reported by other studies [Lebel *et al*. 1996; Auzoux-Bordenave *et al*. 2007]. Rounded epithelial/amoeboid cells are visible (A) as well as fibroblast-like cells (B). Spindle-shaped (C) cells emanate from cell clusters (D).

3.4. Discussion

3.4.1. Gametes

It was found that carrying out fertilizations in 250ml Erlenmeyer flasks instead of culture dishes or 50ml tubes was most practical for the laboratory setup of this study. Erlenmeyer flasks are relatively easily obtainable laboratory glassware and standard in most laboratories.

Erlenmeyer flasks are easily cleaned and are autoclaveable and therefore re-useable. The Erlenmeyer flask's shape makes it spatially economical, manoeuvrable, and easily stacked in an incubator and less likely to spill its contents.

The process of collecting gametes and thereafter carrying out fertilizations was simplified by doing this on a commercial farm where spawning is routinely carried out every week. It would be an arduous and costly task to attempt the acquisition, housing and spawning of sufficient numbers of mature abalone to complete a study such as this one in an acceptable time-frame. The transport of gametes is however not feasible due to the limited life-span of gametes as well as the deleterious effects temperature and environmental changes have on the viability of gametes. Therefore, the construction of a fully-equipped on-site laboratory contributed to the practicality of this study. Even though an on-site laboratory promoted the housing of larvae, larvae could still only be housed for less than 7 days. The settling of larvae could not be carried out in the laboratory environment due to the large volumes of aerated FSW, substrate (diatoms) and settling densities necessary for the long-term survival of settled spat. Meeting the biological requirements of larvae and settled spat was further complicated by strict GMO regulations regarding containment of possible GM larvae and contaminated material.

Optimal fertilization was obtained with gametes fertilised in a 250ml Erlenmeyer containing 200ml FSW with $1.0x10^3$/ml ova and $5.0x10^7$ sperm cells. These results correspond to that found by studies done on other abalone species [Baker and Tyler 2001; Grubert *et al*. 2005]. These fertilization paramaters for *H. midae* implies a ova:sperm ratio of 1:5000, with a sperm concentration of $2.5x10^5$ sperm/ml. The ratio of ova to sperm (1:5000) is however much higher than the ratio of 1:100 to 1:500 reported by Taylor and Baker *et al.* (2001) for *H. tuberculata*. Wang *et al*. (2004) however, reported the ratio of 1:10 000 in *H. discus hannai.* A species specific difference in the sperm to ova ratio might be the cause, although the influence of different experimental procedures cannot be excluded as possible causes for the observed difference. The sperm concentration we propose for optimum fertilization is similar to those reported by other abalone studies. Several species are reported to have an optimum fertilization at a sperm concentration of between 10^5 to 10^6 sperm/ml [Leighton and Lewis 1982; Mill and McCormick 1989; Gao *et al*. 1990; Clavier 1992; Baker and Tyler 2001]. Other

species such as *H. laevigata* and *H. rubra* are reported to exhibit optimum fertilization at a sperm concentration of $2x10^5$ sperm/ml [Fallu 1991], while *H. asinina* has a concentration of $5x10^3$ to 10^5 sperm/ml [Encena *et al*. 1998]. The sperm concentration we found to be necessary for optimum fertilization in *H. midae* is therefore within the range of those observed for other haliotid species.

The fact that gametes were more viable within two hours of spawning is also to be expected at the hand of other reports suggesting the same for other abalone species [Encena *et al*. 1998; Baker and Tyler 2001].

Elucidation of the fertilization parameters of *H. midae* makes it possible to employ ova, sperm, fertilized ova, larvae or a combination of these as targets for gene transfer. Sperm has been used successfully as target tissue and can transmit transgenes to the ova during fertilization [Tsai *et al*. 1997; Sin *et al*. 1995; Lavitrano *et al*. 2006]. From our early initial studies it would seem that sperm cells or early larval stages are prime targets for gene transfer. In comparison with ova, the sperm cells are more robust and remain viable for a much longer period (personal observation). The longer viability affords researchers time to execute the transfection protocol. Male abalone usually spawn before female abalone (in a commercial farm environment), making sperm cells available before ova, also allowing researchers time for transfection before sperm is used for fertilization.

Employing fertilized ova or early larval stages as targets for gene transfer could also be plausible. There are however certain drawbacks; to prevent the creation of transfection mosaics, transfection will have to take place before the first cell division. In *H. midae*, twenty minutes elapses from the first sperm cell entering the ova and fertilizing it, to the first cell division [Arai *et al*. 1986]. This would give researchers a period of only 20 minutes to carry out a transfection protocol. Further practical considerations complicate the procedure: it should be kept in mind that samples would contain numerous ova being fertilized. Each of these ova would have a varying timescale for fertilization, making it difficult to determine when 20 minutes for transfection would start and thereby also shortening the possible 20 minutes to perform the transfection protocol in. Fertilized ova are similarly as sensitive to environmental changes as ova are. A chemical transfection protocol causes fertilized ova environmental,

physical and structural stress. All of these factors contribute to the cessation of further development for individual larvae as well as the abnormal development and death of larvae. Therefore, making use of fertilized ova that have proceeded to cell division and that consist of several cells would be more practical due to their more robust nature as well affording researchers a longer period to perform transfection in. Making use of the multi-cellular larvae would however, result in a much higher frequency of mosaics [Gomez-Chiarri 1998; Sun *et al*. 2005].

The execution of a successful gene transfer strategy centred on gamete, fertilized ova or larvae would have to take all these factors into account. However, several other factors, such as cytotoxicity of transfection reagents, transfection efficiency and expression of the transgene also needs to be taken into consideration and will be investigated in further chapters.

3.4.2. Cell culture

The bleeding of animals for the collection of haemolymph was more practical than the extraction of haemolymph by hypodermic needle. Animals that were bled could be transported under controlled conditions to laboratory facilities suitable for haemolymph collection and culture. Animal used for extraction *via* hypodermic needle needed to remain at abalone farms. Due to the fact that haemolymph adheres to polypropylene and glass surfaces and that this process takes place rapidly after the haemolymph leaves the animal, haemolymph could not be extracted from farm abalone and be transported to laboratory facilities.

The culture of mantle, abductor muscle en epipodocyte tissue yielded poor results. This result might have been expected due to the lack of studies pursuing culture of abductor muscle and epipodocyte tissue as well as the fact that the culture protocol was not optimized with respect to the requirements of these tissues. This is however not true for the mantle tissue cultures where previous studies have had success in culturing this type of tissue (albeit not as successfully as published results suggests – personal communication with Mathilde Van der Merwe). Contamination or inefficacy of reagents, media, plasticware and laboratory equipment could be excluded as possible reasons for the poor cell quality and growth exhibit

by the mantle cultures because haemocyte culture was conducted with exactly the same reagents, media, plasticware and laboratory equipment. The stringent antibiotic decontamination washes and overnight incubation with a relatively strong antibiotic cocktail might be to blame for the inability of mantle explants to produce quality growing cells in culture. Experimentation with the effect of transfection reagents on abovementioned tissue cultures as well as transfection procedures cannot be carried out until successful culture protocols are developed for these tissues.

Haemolymph culture yielded favourable results with haemocytes exhibiting rapid attachment to culture wells, the formation of pseudopodia, cell networks, the presence of identifiable cell types and viability of at least a week. Although no proliferation could be observed, the fact that the haemocytes remained viable for at least a week warrants further investigation into the effect of transfection reagents on these cultures as well as transfection studies. It has to be noted however, that other studies of primary mollusc cell cultures have reported cells to be in G0/G1 phase and therefore not actively cycling and proliferating, causing concerns for these tissues to actively express possible transgenes. Auzoux-Bordenave *et al*. (2007) found only 8% of mantle cell culture to be actively dividing (mantle cells are known to proliferate while haemocytes not). The authors comment that this is not surprising and that this was also seen in other mollusc studies [Rinkevich 1999, 2005]. Boulo *et al*. (2000) proposed the transfection (infection) of cultured Pacific oyster heart tissue and dissociated embryo tissue by pantropic retroviral vectors. To determine the optimum timing for transfection (retroviral vector infection), the mitotic activity, a measure of cell division, was measured. Less than 5% of heart cells were exhibiting mitotic activity while 50% of the dissociated embryo cultures had mitotic activity. Due to this result, Boulo *et al*. (2000) abandoned the heart cells as target tissue. Lebel *et al*. (1996) also stated cell proliferation as a main concern in abalone cell culture.

3.4.3. Future research

Future research should centre on the creation of a proliferative permanent abalone cell line suitable for transfection studies, as well as investigations into other tissues as possible transfection target. The creation of a proliferative permanent cell line for abalone, would not

only boost transfection studies, but would also benefit studies on cell signalling, immune response, disease resistance and pearl-forming studies, just to name a few.

Direct-testis injection could offer a relatively simple approach to creating sperm carrying transgenes which can then be used for fertilization. This approach has been followed in *H. diversicolor supertexta* as reported on by Chen *et al*. (2006). More than 60% of juveniles produced from this technique contained the transgene. *In vitro* transfection where the transgene and transfection reagent is injected into the sinus of adult Eastern oysters has been carried out by Buchanan *et al*. (2001). Haemolymph extracted from treated individuals contained the transgene for at least 4 days after extraction. Both these approaches could be invested in for alternate target tissue approaches and would simplify existing protocols.

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4. Cytotoxicity

4.1. Introduction

Transfection by chemical means requires that target tissues be in contact with several possibly cytotoxic substances. Due to the nature of chemical transfection, target tissue will be treated with transfection reagents, foreign DNA and most likely selection agents such as antibiotics. It is known that increasing the concentration of transfection reagent and foreign DNA increases the possible number of cells that are transfected. However, there is a limit to the extent that foreign DNA and especially transfection reagents can be increased. By increasing the amount of transfection reagent and foreign DNA added to cells, the cytotoxic effect these reagents possibly have on the cells also increases. At a certain threshold the advantage of increasing the amount of transfection reagent and foreign DNA, thereby increasing the number of transfected cells, is mirrored by the cytotoxic effect of the transfection reagents and/or foreign DNA. At this point, increasing the transfection reagent and foreign DNA concentration would lead to an overall lower yield of transfected cells due to the cytotoxic effect of the transfection reagents and foreign DNA. Consequently, it is of great importance to know what the cytotoxic potential of transfection reagents and foreign DNA is to make it possible to find an optimum between increased transfection and minimum cytotoxicity resulting in the highest yield of transfected cells possible [Buchanan *et al*. 2001; Erhardt *et al*. 2006; Huh *et al*. 2007].

In an attempt to select for transfected cells, a selection agent such as antibiotics, to which only transfected cells carrying the foreign DNA are resistant, is added to cells. Adding excessive concentrations of antibiotics will however result in even transfected cells being destroyed or inhibited. The addition of excessively high concentrations of antibiotics also puts stress on transfected cells that could result in the demise of the transfected cells or degeneration of the cells, higher mutation rates and the expulsion of the foreign DNA (plasmid). It is of utmost importance to ensure the use of the antibiotic at its minimum lethal concentration; the lowest concentration at which there is significantly less cell survival. To achieve this, cells that will undergo selection by an antibiotic needs to be treated with a concentration series of this antibiotic to determine the lowest concentration at which significantly less cell survival is observed [Buchanan *et al*. 2001].

In this study two commercially available sources of the molecule polyethylenimine (PEI) was used for transfection; crude branched 25kDa PEI obtained from Sigma-Aldrich and a trademarked transfection reagent, ExGen 500 from Fermentas. Target tissues will therefore be in contact with these transfection reagents as well as the foreign DNA, the pTracer and DsRed plasmids, and the antibiotics, zeocin and neomycin, used for selection of transfected cells.

4.1.1. PEI

PEI is an organic polymer of which every third atom is a protonable amino nitrogen atom, making PEI the organic macromolecule with the highest cationic-charge-density potential. Boussif *et al*. (1995) found PEI to be almost non-toxic when used in transfection. Boussif *et al*. (1995) added that PEI has been widely used in a variety of applications such as water purification, mineral extraction and even in shampoos. However, their study also noted that in cell cultures some toxicity could be observed at concentrations of PEI twice as high as necessary for optimal transfection. Although the exact mechanism for the cytotoxicity caused by PEI is unknown, Horbinski *et al*. (2001) postulated it to be due to the disruption of the endosome/lysosome complexes in cells.

4.1.2. ExGen 500

ExGen 500 is a linear 22kDa PEI molecule produced by Fermentas and reported to be minimally cytotoxic [Ferrari 1997]. ExGen 500 was most efficient at transfecting the majority of cell types tested by Gebhart and Kabanov (2001). When the cell density was increased, the efficiency of ExGen 500 also increased, with a concurrent decrease in cytotoxicity. Although ExGen 500 was shown to be the most efficient transfection reagent, branched PEI of 25kDa and 50kDa, although less active, was less cytotoxic. Smith *et al.* (2003) also reported on the possible genetic toxicity of certain transfection reagents and concluded that ExGen 500 could cause mutations to cells at high concentrations.

4.1.3. Zeocin

Zeocin is a bleomycin/phleomycin antibiotic isolated from *Streptomyces verticillus* that binds and cleaves DNA within cells. Resistance to zeocin is inferred by the *ble* resistance gene that codes for proteins that bind to zeocin and inhibits its strand cleavage activity. Zeocin has

been found to be effectively toxic to bacteria, fungi, plant and mammalian cells [Gatignol *et al*. 1987; Mulsant *et al*. 1988; Perez *et al*. 1989; Drocourt *et al*. 1990; Calmels *et al*. 1991].

4.1.4. Neomycin

Resistance to neomycin, an inhibitor of protein synthesis, is conferred by the aminoglycoside phosphotransferanse II gene. Although this gene is of bacterial origin, it is expressed effectively in eukaryotic cells and confers resistance to neomycin antibiotics in these cells [Buchanan *et al*. 2001]. Neomycin resistance is commonly used for *in vitro* selection of transformed cells and has been expressed in amongst others, species of mice [Kaur *et al*. 1998], fish [Szelei *et al*. 1994] and oysters [Buchanan *et al*. 2001].

Buchanan *et al*. (2001) found concentrations of 0.3mg/ml of neomycin to be sufficiently toxic to *Crassostrea virginica* (eastern oyster) embryos to make selection of individuals carrying a *neo*^{r} transgene possible. The authors also noted that concentrations of 1.0 mg/ml neomycin led to a pH decrease of 8.0 to 7.0 and that the use of neomycin above a concentration of 1mg/ml was therefore not recommended

4.1.5. Plasmids

The 6.2kb pTracer-CMV2 plasmid includes an ampicillin resistance gene, a zeocin resistance gene fused to a Cycle 3-GFP, a cytomegalovirus (CMV) promoter and a multiple cloning site. This construct has been successfully used in *Haliotis* by Wang *et al*. (2004). The ampicillin gene is used for selection of *E. coli* colonies whereas zeocin serves as a selection agent for both prokaryotic and eukaryotic cells. The pCMV-DsRed-Express vector contains a neomycin/kanamycin resistance cassette with promoters for expression in bacteria and mammals (eukaryotes). The DsRed-Express gene is under regulation by the CMV immediate early promoter (see Figure 5.1 for detailed construct design).

It is most beneficial if the transfection reagent and plasmids are as non-cytotoxic as possible, while the selection agents (antibiotics) are cytotoxic and cause cell death or degeneration at reasonable concentrations. To determine if this was the case, to assess the suitability of the reagents for transfection studies and to determine the concentration where these reagents are significantly cytotoxic, the target tissues for transfection in *H. midae* were treated with

these reagents. The target tissues were treated with the reagents in the manner, time-frame and target's developmental stage as would be necessary for transfection studies. Therefore, sperm, fertilized ova and haemocytes were treated with ExGen 500, 25kDa PEI and the plasmids respectively. Fertilized ova, larvae and haemocytes were treated with the antibiotics zeocin and neomycin.

4.2. Methods and materials

4.2.1. Gametes

4.2.1.1. ExGen 500 and 25kDa PEI preparation

The cytotoxicity of the transfection reagent, ExGen 500 (Fermentas) and the 25kDa PEI (Sigma-Aldrich), was determined by adding a series (see Table 4.2 to Table 4.5) of the reagents to sperm and fertilized ova and using the treated sperm for fertilization, subsequently determining the amount of normal larvae obtained from these fertilizations and treated fertilized ova. ExGen 500 was prepared according to the manufacturer's specifications by adding ExGen 500 to 100µl of a 150mM NaCl solution and incubating the solution for 10 minutes before addition to the gametes. The 25kDa PEI was obtained from Sigma-Aldrich (Catalogue number: 408727) as a highly viscous liquid. This solution was aliquoted, diluted to a stock concentration of 5mg/ml or 20% (v/v) which was then neutralized by the addition of HCl to achieve a pH of 7.5, filter sterilized and stored at -20ºC. The process of aliquoting and diluting the viscous liquid was more efficient when it was heated slightly by immersing the container in tepid water.

4.2.1.2. Sperm

One millilitre of sperm at a concentration of 2.15×10^6/ml was added to the transfection reagents (ExGen 500 and the 25kDa PEI) and incubated for 1 hour at 20℃ in a polypropylene eppendorf tube, after which half the samples were directly used to fertilize untreated ova. The other half of the treated sperm samples underwent centrifugation (1 minute at 5000g) to form a pellet and was resuspended in 1ml FSW. These sperm cells were also used to fertilize untreated ova. Both treatment groups were then left to hatch overnight at 18℃ in 6-well plates (Cellstar, Greiner Bio-one) containing 8ml of FSW before the number of normal and abnormal larvae was counted. Control samples underwent the same procedure

as treatment groups, with the exception of the addition of ExGen 500 or 25kDa PEI; FSW was added instead.

4.2.1.3. Ova

Fertilized ova were obtained from normal production stock 20 minutes post-fertilization at a concentration of $1.0x10^3$/ml. Transfection reagents (ExGen 500 and the 25kDa PEI) were added immediately to half of these samples (1ml) and the mixture incubated for 2 hours at 18 to 20℃. For the remaining samples, the transfectio n reagents were only added after 2 hours of incubation at 18 to 20℃. All the fertilized ova were left overnight in 6-well plates (Cellstar, Greiner Bio-one) containing 8ml of FSW and incubated at 18℃ to hatch before the number of normal and abnormal larvae was counted. Controls were not treated with the transfection reagents and FSW was added as a placebo.

4.2.1.4. Plasmids

The pTracer-CMV2 (Invitrogen) and the pCMV-DsRed-Express (Clontech) plasmids were added to 1ml sperm ($2.15x10^6$/ml) immediately after spawning and to 1ml fertilized ova ($1.0x10^3$/ml) immediately after fertilization. A series (1.0µg, 3.0µg, 10µg and 50ug) of the plasmids with a concentration of 1000ng/µl was used. Treated sperm was used to fertilize ova while fertilized ova were left to hatch overnight at 18℃ in 6-well plates (Cellstar, Greiner Bio-one) containing 8ml of FSW before the number of normal and abnormal larvae was counted. Control samples underwent the same procedure as treatment groups, with the exception of the addition of the plasmids; FSW was added instead.

4.2.1.5. Zeocin and neomycin

The biocidal effect of zeocin and neomycin was monitored by adding a concentration series of the antibiotics to fertilized abalone ova. Fertilized ova were harvested and pipetted into 12-well plates (Cellstar, Greiner Bio-one) with zeocin at a concentration of 25µg/ml; 100µg/ml; 500µg/ml and 1000µg/ml and allowed sufficient time to hatch overnight at 18ºC before the percentage of normally developed hatchlings were determined by counting normally developed larvae compared to abnormal larvae. These plates were covered to prevent zeocin's efficacy being compromised due to light exposure. The same was done with a

concentration series of neomycin (25µg/ml; 100µg/ml; 200µg/ml; 400µg/ml; 600µg/ml; 800µg/ml; 1000µg/ml and 1200µg/ml).

Another group of fertilized eggs were allowed to hatch in a 2 litre glass container overnight, after which living hatchlings were harvested and pipetted into 6-well culture plates (Cellstar, Greiner Bio-one) to which a concentration series of zeocin was added at concentration of 1000µg/ml; 100µg/ml; 25µg/ml. In control samples, zeocin was substituted with FSW.

4.2.2. Cell culture (haemocytes)

4.2.2.1. ExGen 500 and 25kDa PEI

The cytotoxic effect of ExGen 500 (Fermentas) and the 25kDa PEI (Sigma-Aldrich) on haemocyte cell culture was examined by treating haemocyte cultures with a concentration series of ExGen 500 and 25kDa PEI respectively and then determining the relative survival by quantification of the viability of cells using a colorimetric assay (XTT). Haemocytes were seeded as previously described (see Chapter 3: Target tissue) and incubated at 18ºC for 2 hours before the culture medium was removed and ExGen 500 and the 25kDa PEI was added (see Table 4.1). After addition of the ExGen 500 and the 25kDa PEI, the plates were incubated for 5 minutes at room temperature and agitated to ensure that the reagents spread across each well sufficiently before the culture medium was replaced (100µl/well). ExGen 500 and 25kDa PEI were not removed before addition of the fresh culture medium. Plates were incubated at 18ºC overnight. The next morning the culture medium was once again replaced with fresh culture medium and 50µl of XTT reagents (Roche) were added to each well and allowed to incubate for 6 hours at 18ºC before taking an absorbance reading with an ELISA plate reader (Bio-Rad). Living cells metabolise the yellow XTT reagent to an orange formazan solution. An increase in absorbance readings are therefore in accordance with an increase in orange formazan and are therefore directly proportional to the number of living cells. Plates were kept covered to ensure minimal light exposure during incubation (XTT reagents are light-sensitive).

Table 4.1: The volume of ExGen 500 and 25kDa PEI added to haemocyte cell cultures in an attempt to determine the cytotoxic effect of these transfection reagents.

Volume of ExGen 500 and 25kDa PEI (µl)
0
0.8
0.990
1.15
1.32
1.48
2

(Volumes are representative of only the ExGen 500 added to each sample and does not include the 150mM NaCl solution added to the ExGen 500)

4.2.2.2. Plasmids

The same aforementioned protocol as the one followed for the transfection reagents, ExGen 500 and the 25kDa PEI, was followed to determine the possible cytotoxic effect of the pTracer-CMV2 and the pCMV-DsRed-Express plasmids on haemocyte cell culture. A series (0.1µg, 0.3µg, 0.5µg and 1.0µg) of both plasmids were added to one day old cultured haemocytes.

4.2.2.3 Zeocin and neomycin

The cytotoxic effect of zeocin and neomycin on haemocyte cell culture was examined by treating haemocyte cultures with a concentration series of zeocin and neomycin respectively and then determining the relative survival of samples by quantification of the viability of cells using a colorimetric assay (XTT). Haemocytes were once again seeded as previously described (see Chapter 3: Target tissue) and incubated at 18ºC for 2 hours before the culture medium was removed and zeocin or neomycin was added (see Table 4.7). After addition of the relevant antibiotic the plates were incubated for 5 minutes at room temperature and agitated to ensure that the reagents spread across each well sufficiently before the culture medium was replaced (100µl/well). Zeocin or neomycin was not removed before addition of the fresh culture medium. Plates were incubated at 18ºC overnight in the dark. The next morning the culture medium was once again replaced with fresh culture medium and 50µl of

XTT reagents (Roche) were added to each well and allowed to incubate for 6 hours at 18ºC before taking an absorbance reading with an ELISA plate reader.

All experiments were carried out using three biological replicates containing three technical replicates each.

4.2.3. Data analysis

An analysis of variance (ANOVA) was performed on data to determine whether there was a difference in variance between the treatment groups. If a p-value of less than 0.05 was attained a significant difference in variance between the treatment groups was present and an F-test was performed to determine whether the variance of the control group compared with each treatment group was equal (p-value > 0.05) or unequal (p-value < 0.05) to determine the nature of t-test that would be performed. The t-test compared the mean of the control group with that of each of the treatment groups where a p-value < 0.05 indicated there to be a significant difference between the two groups while a p-value > 0.05 indicated that there was no significant difference between the two groups.

4.3. Results

4.3.1. Gametes

4.3.1.1ExGen 500

An ANOVA was performed using the data obtained from the number of normally hatched larvae after treatment of the fertilized ova with ExGen 500 (Table 4.2). This analysis indicated that there was no significant difference between the treatment groups (p-value > 0.05).

Table 4.2: The volume of ExGen 500 added to fertilized ova and the resulting mean percentage of normally developed larvae.

Volume of ExGen 500 (µl)	Percentage normal larvae	Standard deviation
Fertilized ova exposed to ExGen 500 two hours after fertilization		
0	95.31	1.52
2.75	90.41	2.43
3.30	86.97	5.87

4.30	91.20	2.60
4.90	87.67	4.34
Fertilized ova exposed to ExGen 500 immediately after fertilization		
0	86.97	5.05
2.75	79.26	4.73
3.30	85.94	4.12
4.30	85.00	1.91
4.90	80.14	2.39

There was no significant difference (p-value > 0.05) between any of the treatment groups after treatment of sperm with ExGen 500 (Table 4.3).

Table 4.3: The volume of ExGen 500 added to sperm and the resulting mean percentage of normally developed larvae.

Volume of ExGen 500 (µl)	Percentage normal larvae	Standard deviation
Sperm exposed to ExGen 500 and centrifuged for removal of ExGen 500 before fertilization		
0	62.06	1.52
2.75	57.86	23.37
3.30	70.43	6.68
4.30	52.58	2.30
4.90	69.49	2.52
Sperm exposed to ExGen 500 without removal of ExGen 500 before fertilization		
0	66.30	5.46
2.75	65.10	8.20
3.30	65.88	15.23
4.30	48.82	6.39
4.90	53.80	12.79

4.3.1.2. 25kDa PEI

There was a significant difference (p-value < 0.05) in the number of normal larvae between treatment groups after addition of 25kDa PEI to fertilized ova two hours after fertilization. A t-test indicated there to be significant differences between all the treatments groups and the control, except the first treatment where the least amount of 25kDa PEI (2.75µl) was added. There was no significant difference (p-value > 0.05) for the treatment groups to which the 25kDa PEI was added immediately after fertilization (see Table 4.4).

Table 4.4: The volume of 20% (v/v) 25kDa PEI added to fertilized ova and the resulting mean percentage of normally developed larvae.

Volume of 20% (v/v) 25kDa PEI (µl)	Percentage normal larvae	Standard deviation
Fertilized ova exposed to 25kDa PEI two hours after fertilization		
0	68.87	1.92
2.75	53.50	18.70
3.30	43.39	13.77
4.30	29.39	27.47
4.90	14.06	6.01
Fertilized ova exposed to 25kDa PEI immediately after fertilization		
0	67.86	11.22
2.75	34.99	15.39
3.30	31.93	13.28
4.30	22.79	18.32
4.90	24.80	9.41

There was a significant difference (p-value < 0.05) in the number of normal larvae from fertilizations carried out with sperm that underwent 25kDa treatment and centrifugation. A t-test indicted there to be significant differences between all of the treatment groups and the

control. There was no significant difference (p-value > 0.05) in treatment groups when sperm was not centrifuged (see Table 4.5).

Table 4.5: The volume of 20% (v/v) PEI added to sperm and the resulting mean percentage of normally developed hatchlings from the replicates.

Volume of 20% (v/v) 25kDa PEI (µl)	Percentage normal hatchlings	Standard deviation
Sperm exposed to 25kDa PEI and centrifuged for removal of PEI before fertilization		
0	66.47	11.46
2.75	41.43	20.16
3.30	19.74	4.81
4.30	16.15	13.16
4.90	16.75	7.51
Sperm exposed to 25kDa PEI without removal of PEI before fertilization		
0	17.83	13.38
2.75	9.92	3.61
3.30	7.72	2.09
4.30	13.61	6.84
4.90	11.57	9.46

4.3.1.3. Plasmids

An ANOVA indicated there to be no significant difference between larval survival after treatment of fertilized ova and sperm with the plasmids (p-value > 0.05).

4.3.1.4. Zeocin and neomycin

An ANOVA provided a p-value of < 0.05, indicated there to be a significant difference between the respective zeocin and neomycin treatment groups. A t-test was performed comparing the mean of the control group with each of the treatment groups (see Table 4.6). The p-values indicated a significant difference between the control and the treatment groups

for zeocin concentrations of 100µg/ml to 1000µg/ml and for neomycin concentrations between 800µg/ml to 1200µg/ml.

Table 4.6: Larvae were exposed to a concentration series of zeocin and neomycin. The percentage larval survival was calculated for each concentration. A t-test was performed to assess the difference between each treatment group and the control group.

Concentration of zeocin (µg/ml)	Percentage hatchling survival	p-value	Standard deviation
Control	30.60	N/A	9.45
25.00	17.94	0.13	1.73
50.00	16.73	0.07	3.6
100.00	6.91	0.02	4.36
500.00	2.00	0.03	1.15
1000.00	1.00	0.03	0.58
Concentration of neomycin (µg/ml)	Percentage hatchling survival	p-value	Standard deviation
Control	30.60	N/A	9.45
25.00	33.73	0.73	5.29
100.00	30.00	0.79	1.00
200.00	28.81	0.68	4.00
400.00	27.77	0.64	6.65
600.00	26.54	0.52	6.24
800.00	4.16	0.04	1.52
1000.00	2.29	0.03	0.58
1200.00	1.41	0.01	1.00

The group of fertilized ova that was allowed to hatch overnight and treated with zeocin the next morning did not exhibit any negative effects due to the biocidal effect or toxicity of zeocin. This continued for 8 hours until the experiment was halted because it was established that laboratory conditions such as a lack of oxygenated FSW, instead of the effect of zeocin,

was starting to play a role in larval survival. This was established by monitoring controls where no zeocin had been added and where larvae were housed under identical conditions to experimental groups. This experiment was repeated and the same was observed with the experiment having to be halted due to environmental conditions.

4.3.2. Cell culture (haemocytes)

4.3.2.1. ExGen 500 and 25kDa PEI

There was no significant difference between groups after treatment with ExGen 500 and 25kDa PEI respectively (p-value > 0.05). The p-value for the ExGen 500 was found to be equal to 0.769 and for the 25kDa PEI to be equal to 0.933.

4.3.2.2. Plasmids

An ANOVA was performed using the data obtained from the XTT results. The p-value was found to be > 0.05 for both plasmids with the p-value for the pTracer-CMV2 plasmids being equal to 0.790 and for the pCMV-DsRed-Express being equal to 0.968, indicating that there to be no significant difference between treatment groups.

4.3.2.3. Zeocin and neomycin

The p-value obtained from the ANOVA for zeocin and neomycin was < 0.05 (0.001 and 0.011). The t-test indicated there to be significant differences for the addition of zeocin at a concentration of 50µg/ml or more. In the case of neomycin, the only treatment group that showed a significant difference was the 500µg/ml treatment. This is an unexpected result; especially considering that treatments that had higher neomycin concentrations did not yield a significant difference. This experiment was repeated another two times with no significant difference being observed between any of the treatment groups. Other factors are most probably responsible for this result (see Table 4.7).

Table 4.7: The volume of neomycin and zeocin added to haemocyte cultures and the resulting p-value obtained from a t-test from relative survival data.

Volume of zeocin added (µg/ml)	p-value	Standard deviation
Control	N/A	0.03
25.00	0.10	0.05
50.00	0.01	0.05
100.00	0.00	0.03
500.00	0.00	0.17
1000.00	0.00	0.03
Volume of Neomycin added (µg/ml)	**p-value**	**Standard deviation**
Control	N/A	0.08
10.00	0.01	0.06
50.00	0.07	0.04
100.00	0.27	0.12
150.00	0.11	0.10
500.00	0.01	0.04
1000.00	0.14	0.28
2000.00	0.98	0.15
5000.00	0.05	0.04

4.4. Discussion

4.4.1. Gametes

4.4.1.1. ExGen 500

In spite of the fact that the $1.0x10^3$ fertilized ova treated with ExGen 500 underwent treatment at more than 3 to 4 times the recommended amount of ExGen 500 according to the manufacturer's specifications (determined by the number of cells), there was no significant

difference between treatments to indicate that ExGen 500 had a negative effect on normal hatching and survival of fertilized ova. Also, the length of exposure to ExGen 500 and the point at which ExGen 500 was added (immediately after fertilization or 2 hours after fertilization) did not influence the normal development and hatching of larvae.

It also seems that ExGen 500 does not have a negative effect on the fertilization potential of sperm or the development and normal hatching of larvae resulting from fertilizations by treated sperm. Both the treatment groups, the sperm that was centrifuged to remove ExGen 500 before fertilization and the treatment group without removal of ExGen 500, did not exhibit any significant differences in larval survival. It can therefore be assumed that the presence of ExGen 500 at the time fertilization takes place is also not detrimental to normal development or larval survival.

It is therefore concluded that ExGen 500 does not have a cytotoxic effect on sperm or fertilized ova at these concentrations and under these conditions and could therefore serve as a potential transfection reagent.

4.4.1.2. 25kDa PEI

ANOVA results obtained from the number of normally hatched larvae after treatment of fertilized ova or sperm with the 25kDa PEI indicated there to be differences between treatments in some of the treatment groups. A significant difference in normal hatchling survival was noted for the treatment group to which 25kDa PEI was added 2 hours after fertilization. A t-test indicated there to be significant differences between all the treatment groups and the control, except for the first treatment where the least amount (2.75µl) of the 25kDa PEI was added. No significant difference in the number of normally hatched larvae for the group treated with the 25kDa PEI immediately after fertilization was found. Therefore the significant differences in treatment groups for fertilized ova treated with the 25kDa PEI might be due to the time-point at which the PEI was added seeing that the experimental group to which the 25kDa PEI was added immediately after fertilization did not exhibit any significant drop in normal larval survival and development. Two hours after fertilization the embryo already consists of several rapidly dividing cells. The actual process of addition of the PEI

rather than the PEI itself might therefore be to blame for the lower hatchling normality and survival.

The same was seen in the data obtained from the sperm treated with the 25kDa PEI; one experimental group exhibited a significant difference in normally hatched larvae while the other group did not. A significant difference for larval survival was seen between all treatment groups of which the sperm was treated with 25kDa PEI and centrifuged to remove the PEI. As in the aforementioned instance the actual treatment procedure instead of the PEI might be to blame for the drop in larval survival. Centrifugation has the ability to damage cells and the compacted state with limited oxygenated FSW, however short, could have a negative effect on the viability of sperm cells. The actual cytotoxic effect of the 25kDa PEI should however not be eliminated as the reason for the lower hatchling survival and normal development. No significant difference in larval survival for any of the treatment groups was observed for sperm treated with the 25kDa PEI and used directly for fertilizations.

From these results it is evident that the 25kDa PEI is not significantly cytotoxic (at the concentrations tested) to fertilized ova immediately after fertilization or to sperm (without centrifugation). The 25kDa should however be used carefully where cells will have to undergo physical rigors during the treatment process or in cases where cells are undergoing critical developmental stages. It should be kept in mind that the amount of the 25kDa PEI used in these cytotoxicity experiments was much higher than those used in other studies for optimal transfection [Erhardt *et al*. 2006] as a means to establish the highest possible concentration of PEI that cells can be exposed to. Lower concentrations of the 25kDa PEI will most probably yield much lower levels of cytotoxicity [Boussif *et al*. 1995; Erhardt *et al*. 2006].

4.4.1.3. Plasmids

No significant difference could be observed between treatment groups treated with a series (1.0µg, 3.0µg, 10µg and 50ug) of the pTracer-CMV2 and the pCMV-DsRed-Express plasmids respectively. It can be concluded that the two plasmids are not significantly cytotoxic to sperm or fertilized ova at the concentrations tested. The high plasmid stock concentration allowed the series of plasmids to be added to the sperm and fertilized ova at relatively low volumes, the largest volume being 50µl. Decreasing the plasmid stock concentration would

correspondingly increase the volume of plasmid being added to gametes and could have a negative effect seeing that the gametes are housed and survive in FSW while the plasmids are diluted in TE buffer (see Appendix for constituents) or distilled water. The significant changes in osmolarity and constituents of the fluid housing the gametes would have a negative effect on their viability and survival.

4.4.1.4. Zeocin and neomycin

Zeocin added to ova immediately after fertilization had a significant effect on hatchling survival and development at a concentration of 100µg/ml or higher. This is an indication that fertilized ova are susceptible to the biocidal effect of zeocin and exhibit significantly lower hatchling survival and normal development at concentrations of 100µg/ml or higher. Zeocin can be considered to be rather effective at relatively low concentrations in fertilized ova considering that 25µg to 50µg for *E. coli*, 50µg to 300µg for yeast and 50µg to 1000µg for mammalian cells are the manufacturer's recommended concentrations.

The concentration of neomycin necessary to significantly influence the survival of fertilized ova, 800µg/ml, is greater than the concentration Buchanan *et al*. (2001) recommended for eastern oyster (*Crassostrea virginica*) larvae. This concentration is however within the range recommended by the manufacturer (50 to 1000µg/ml).

In contrast, hatched larvae that were treated with the same zeocin concentration series did not exhibit the same indication of cell death, stunted and abnormal development, as seen in the other experimental group. It should be noted that larvae were not exposed to zeocin for the length of time that the fertilized ova were, due to suboptimal conditions in the laboratory for long term survival of larvae. Fertilized ova were exposed to zeocin for 18 hours while the hatched larvae were exposed to zeocin for only 8 hours. Increased length of exposure time might therefore have yielded the same results as seen in the fertilized ova group. Larvae are much more robust than fertilized ova and comprise greater protective layers, therefore it can be assumed that an increase in the concentration of zeocin would be necessary to significantly effect larval survival. If larvae are to be treated with zeocin, further experimentation is needed under conditions where hatchlings can be treated with zeocin for

at least 18 hours without laboratory conditions impeding the hatchling survival. Treatment of larvae with neomycin was not attempted due to aforementioned experimental difficulties.

4.4.2. Cell culture (haemocytes)

4.4.2.1. ExGen 500 and 25kDa PEI

There was no significant difference between any of the treatment groups for both ExGen 500 and the 25kDa PEI. It can be assumed that at the concentrations used, neither ExGen 500 nor the 25kDa PEI is significantly cytotoxic to haemocytes and that these two reagents can be used as possible transfection reagents.

4.4.2.2. Plasmids

No significant difference could be observed between treatment groups treated with a series (0.1µg, 0.3µg, 0.5µg and 1.0µg) of the pTracer-CMV2 and the pCMV-DsRed-Express plasmids respectively. It can be concluded that the two plasmids are not significantly cytotoxic for haemocytes at the concentrations tested. As previously mentioned, the high plasmid stock concentration used in this study allowed the series of plasmids to be added to the haemocytes at relatively small volumes. Decreasing the plasmid stock concentration would correspondingly increase the volume of plasmid being added to haemocytes and could affect the osmolarity of the culture's growth medium, thereby negatively influencing cultures. Plasmid stock concentrations should therefore be large enough not to result in osmolarity changes that would influence cultures negatively.

4.4.2.3. Zeocin and neomycin

A significant decrease in cellular survival and metabolism was seen when 50µg/ml or more zeocin was added to haemocytes. This is the minimum concentration of zeocin recommended by the manufacturer's protocol to be sufficiently cytotoxic to cell cultures. Haemocyte cell cultures are therefore especially susceptible to the biocidal effect of zeocin.

An ambiguous and unexpected result was found with the experiments pertaining to the cytotoxic effect of neomycin. The initial experimental results suggested there to be significant differences between experimental groups with the 500µg/ml concentration of neomycin having a significant effect on haemocytes. A second and third replicate of the experiment

however did not indicate there to be any significant difference between treatment groups. The initial result was already unusual considering that the concentration of 500µg/ml yielded a significant difference while higher concentrations did not. Other factors are most probably responsible for this result. It can be supposed that neomycin is not significantly cytotoxic to haemocytes due to the results of the second and third round of experiments. However, the fact that the first experiment yielded at least one treatment that was significant might indicate there to be inaccuracies incorporated into the quantification procedure. The colorimetric assay used to determine the relative survival of cells by quantification of the viability of cells is dependant on the colour reaction and the resulting colour differences between samples to correctly quantify data. If the neomycin influenced the colorimetric assay by increasing the absorbance reading with an increase in concentration, it could explain the unusual results. Buchanan *et al*. (2001) noted the drastic decrease in pH from 8.0 to 7.0 with an increase in neomycin concentration from 0.0mg/ml to 1mg/ml. The Leibowitz L-15 growth medium that was used to culture the haemocytes and to which the XTT reagent was added, displays a change in colour when confronted with extreme pH shifts [Sigma-Aldrich 2010]. Addition of high concentrations of neomycin could have resulted in the growth medium changing colour and influencing the absorbance readings. Further research will therefore need to be undertaken if neomycin is to be used as selection agent. Treatment of cells with a concentration series of neomycin followed by cell staining to indicate viable cells would be feasible. The possible cytotoxic effect of neomycin on haemocytes can therefore not be excluded.

It can be concluded that the two transfection reagents, ExGen 500 and the 25kDa PEI are relatively non-cytotoxic for sperm, fertilized ova and haemocytes at the concentrations tested, although an increased concentration would lead to higher levels of cytotoxicity. The plasmids also seem to be relatively non-cytotoxic at the concentrations used although adding high volumes of plasmid to gametes and haemocyte could change the osmolarity and constituents of the medium and negatively affect the cells. The lowest toxic concentration of zeocin and neomycin for fertilized ova could be determined and can be employed for selection in further research. The concentration of zeocin necessary for haemocyte cell culture could also be determined however, quantification of neomycin has proven to be difficult most probably due

to neomycin being responsible for a pH decrease in cell cultures. Further research using different quantification methods are needed.

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5. Gene transfer

5.1. Introduction

5.1.1. Transgenesis in aquaculture

The first successful gene transfer to fish was reported on by Zhu *et al*. (1985); the human growth hormone gene was injected into the fertilized eggs of goldfish. Since then, several transgenic aquatic animals have been produced [Levy *et al*. 2000; Rasmussen and Morrissey 2007; Cole 2009] by means of diverse gene transfer techniques including microinjection [Cadoret *et al*. 1997; Wang *et al*. 2004], electroporation [Tsai 2000; Tseng *et al*. 2000; Buchanan *et al.* 2001], biolistic bombardment [Gendreau *et al*. 1995] and recently, chemical-mediated gene transfer [Buchanen *et al*. 2000; Wang *et al*. 2004; Sun *et al*. 2005]. Microinjection is the most common method, although it is surpassed by electroporation for convenience and efficiency [Chen *et al*. 2006]. Microinjection is a relatively efficient technique, but is limited in dealing with the large number of gametes and resulting embryos that are spawned by most shellfish species [Chen *et al*. 2006]. Electroporation and chemical gene transfer methods have been the transfection mode of choice for mollusc transfection studies, most probably due to the fact that these techniques make it possible to rapidly treat the large amount of gametes or larvae produced by most mollusc species. As a result of the efficiency and simplicity of chemically-mediated gene transfer, it has gained popularity for use in aquatic species [Sun *et al*. 2005].

5.1.2. PEI in aquaculture

Studies on marine animals using the chemical transfection reagent, PEI, has yielded positive results [Sun *et al*. 2005]. Although PEI transformation studies have not been carried out on *H. midae*, a study was undertaken on another abalone species (*H. discus hannai*) and has proven successful [Wang *et al*. 2004]. Wang *et al*. (2004) employed a 25kDa PEI to transfect *H. discus hannai* gametes with the pTracer-CMV2 construct. These authors transfected fertilized ova by microinjection and PEI transfection. Also, sperm was transfected by addition of naked DNA and PEI/DNA complexes. Treated sperm and ova were used for fertilizations and the resulting larvae were analysed for the presence and expression of the transgenes. The highest success was achieved with sperm treated with a PEI/DNA complex where 13.9% of embryos developed to blastomeres and expressed the transgene. This group was however also the group with the lowest fertilization and survival rate compared to other treatments.

Sun *et al*. (2005) used PEI in the form of jetPEI to deliver foreign genes to shrimp zygotes at the one cell stage. This technique was compared with microinjection and electroporation and found to be superior for hatchling survival rate, transfection efficiency and expression. Sun *et al*. (2005) postulated that the rigors of the electroporation and microinjection procedures resulted in the low survival rate of hatchlings and that using transfection reagents such as PEI, would offer a gentler, non-invasive and therefore more efficient manner of transfection.

Considering the inability of other methods such as microinjection and ballistic bombardment to transfect the large number of eggs that many mollusc species produce and the inefficiency of electroporation and microinjection as a result of physical damage to zygotes, transfection using chemical reagent such as PEI would therefore be preferable.

5.1.3. Sperm-mediated gene transfer

The abovementioned gene transfer techniques, although effective and popular, rely on the addition of chemical and/or external stimuli and are in some cases invasive and harmful to cells. It has been noted that the sperm of several animal species exhibit the ability to spontaneously take up foreign DNA [Spadafora 1998]. Sperm cells can therefore serve as a natural vector for transfection by taking up foreign DNA and transporting it to the ova and thereby facilitating the incorporation of the foreign DNA into the genome of the embryo [Sin *et al*. 1995]. The mechanism of transfer of foreign DNA by sperm to ova seems to be achieved by DNA binding to the sperm head. It is hypothesized that the presence of CD4-molecules on the sperm cell mediates the uptake. Studies have shown that by combining the natural affinity of sperm cells for DNA with conventional gene transfer techniques further facilitates the process whereby the sperm cell can function as a vector and result in the production of transgenic offspring [Spadafora 1998]. Treatment of sperm with naked DNA for gene transfer has been successful in several species, such as cattle [Robl *et al*. 2007]; mice [Huguet and Esponda 2000]; pigs [Lavitrano *et al*. 2003] and rabbits [Kuznetsov *et al*. 2000]. Physical and chemical gene transfer techniques have been applied to enhance the uptake of foreign DNA by sperm cells. Lipofection, polyplex-mediation and electroporation of sperm has been applied to species such as carp, catfish and tilapia [Mueller *et al*. 1992]; roosters [Rottmann *et al*. 1992]; insects [Shamila and Mathavan 1998] and even haliotids [Tsai *et al*. 1997; Wang *et*

al. 2004]. Sin *et al*. (1995) and Tsai *et al.* (1997) electroporated sperm of *H. iris* and *H. diversicolor supertexta*. Sin *et al*. (1995) confirmed the transfer of the transgenes to sperm and Tsai *et al*. (1997) confirmed sixty five percent of trochophore larvae (at least 16 hours post-fertilization) to contain the transgene.

A disadvantage of sperm-mediated transgenesis is that it results in high rates of chimerism and erratic heritability. Low frequencies of integration of transgenes into the genome of the target tissue have been reported for sperm-mediated transgenesis [Niu and Liang 2008]. Transgenes that do not integrate into the genome of the transfected animal persist extra-chromosomally and results in lack of expression and heritability [Gandolfi 2000; Niu and Liang 2008]. This phenomenon was also reported for tiger shrimp (*Penaeus monodon*) [Tseng *et al*. 2000] and for mud loach (*Misgurnus anguillicaudatus*) [Tsai *et al*. 2000]; the transgenes were mosaically distributed in tissues after sperm-mediated gene transfer was used for creation of these transgenics. However, there are still several advantages in using sperm instead of ova as transgenesis vector. The high concentration of sperm that is generated during spawning in haliotid species makes sperm-mediated gene transfer potentially a mass gene transfer method. The small size of sperm cells compared to ova also increases the chances of foreign DNA being internalised, rather than being delivered to other indiscriminate cell areas [Tsai *et al*. 2000].

5.1.4. Flow cytometry

Flow cytometry refers to a process where the physical and chemical characteristics of biological cells are measured while these cells travel in a fluid stream passing the sensor of a measuring instrument. Flow cytometry utilizing fluorescence measurements was introduced in the late 1960's and today, in the 21st century, most cytometry involves the use of fluorescence measurements [Shapiro 2003]. Compared to fluorescent microscope analysis, flow cytometry has several advantages; the possibility of cells undergoing photobleaching due to extended periods of excitation and emission is eliminated as cells all undergo the exact same length of excitation as they pass rapidly through the flow cytometry instrument. The process of cell sorting or determination of the number of cells expressing a fluorescent protein or containing some other identifiable characteristic is completed with a higher degree of precision and speedier than would be possible utilizing microscopy analysis [Shapiro 2003].

Flow cytometry has been employed to investigate and characterize the haemocytes of mollusc species such as the clams, *Ruditapes philippinarum* and *Mercenaria mercenaria,* also the oyster, *Crassostrea virginica* [Allan *et al*. 2002; Hegaret *et al*. 2003]. Flow cytometry has also been applied to mollusc haemocytes in an attempt to characterize the response of these cells to certain stress situations, such as sudden temperature elevations, as well as their response to parasite challenges [Hegaret *et al*. 2003; Lambert *et al*. 2003].

Flow cytometry has proven to be extremely useful in monitoring expression of fluorescent transgenes [Hawley *et al*. 2004]. Flow cytometry analysis has been employed in several studies to confirm expression of the fluorescent transgene, assess transfection efficiency or discriminate between different fluorescent cell populations [Hawley *et al*. 2001; Nagy *et al*. 2003; Kantakamalakul *et al*. 2003; Halweg *et al.* 2005]. Flow cytometry has also been employed to assess the efficiency of transfection in molluscs. Buchanan *et al*. (2001) used flow cytometry to detect expression of GFP by Eastern oyster (*Crassostrea virginica*) haemocytes.

The current study aims to optimize a transfection protocol for *H. midae* gametes, larvae and haemocyte cells in culture by *in vitro* transfection using PEI in the form of ExGen 500 and a 25kDa PEI with two different plasmids; pTracer-CMV2 and pCMV-DsRed-Express. Integration and expression of the fluorescent transgenes, contained in the plasmids, will by assessed by microscopic analysis in gametes, larvae and haemocytes and flow cytometry in haemocytes. PCR will be employed to confirm the presence and integrity of the EGFP gene (contained in the pTracer-CMV2 plasmid) in larvae.

5.2. Materials and methods

5.2.1. Plasmid preparation

The pTracer-CMV2 (Invitrogen) and pCMV-DsRed-Express (Clontech) (Figure 5.1) were chosen for transfection studies because both the plasmids have been proven to be expressed in eukaryotic cells and Wang *et al*. (2004) employed the pTracer-CMV2 construct for transfection of *H. discus hannai* gametes. Chemically competent *Escherichia coli* cells were transformed using a heat shock method. Transformed cultures were streaked out on LB

medium (see Appendix for constituents) containing the appropriate selection antibiotic; ampicillin at 100µg/ml for selection of cells containing the pTracer-CMV2 construct and kanamycin at 50µg/ml for selection of cells containing the pCMV-DsRed-Express construct. Single colonies were grown overnight in liquid medium containing the appropriate antibiotic and used in extractions using the Qiagen Endofree Plasmid Maxi kit according to the manufacturer's specifications. Purified plasmid was dissolved in TE buffer (see Appendix for constituents) and stored at -4℃.

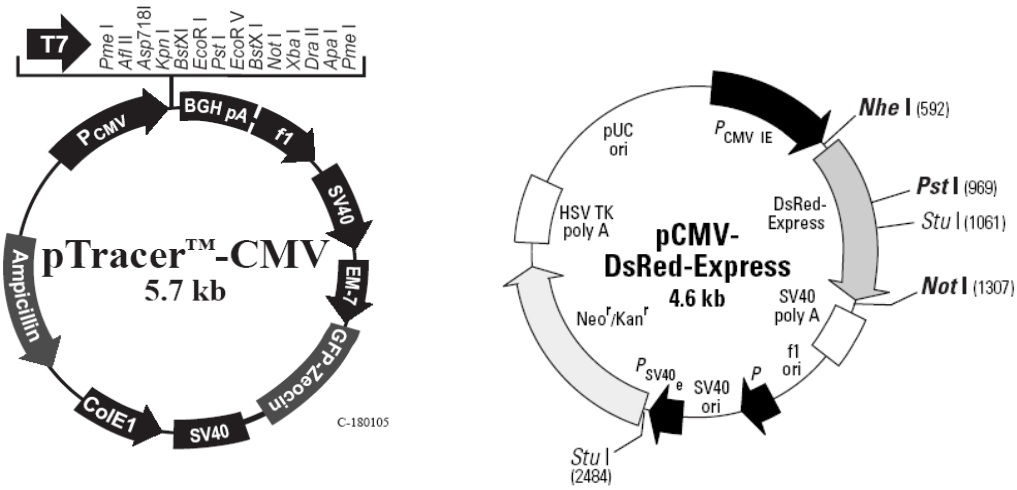

Figure 5.1: The pTracer-CMV2 (Invitrogen) (EGFP) and pCMV-DsRed-Express (Clontech) (DsRed) constructs employed in transfection.

The plasmids were linearized using restriction enzymes that possess only a single cut site on the plasmid. *Eco*R I (Sigma-Aldrich) was used in the case of pTracer-CMV2, while *Ava* II (Sigma-Aldrich) was used for the pCMV-DsRed-Express plasmid. After the digestion reaction, linearized fragments were purified of enzymatic reagents and contaminants using the Qiagen QIAquick Gel Extraction kit according to the manufacturer's specifications, after which fragments underwent agarose gel electrophoresis on a 1% (w/v) agarose gel in 1X TBE (see Appendix) electrophoresis buffer containing 0.05mg/ml Ethidium Bromide to determine whether the digestion reaction did indeed yield linearized fragments. Samples were visualized using the MultiGenius BioImaging System (Syngene). Purified linearized plasmid was dissolved in TE buffer and stored at -4℃.

5.2.2. Transfection of gametes and larvae

Sperm, ova, and larvae were collected as previously described (see Chapter 3: Target tissue). Transfection of sperm, ova and larvae was carried out in 2ml eppendorf tubes before gametes were used for fertilization and fertilized ova and embryos were transferred to Erlenmeyer flasks for hatching. Transfection was carried out by adding transfection reagents to sperm, ova and larvae were in the 2ml eppendorf tubes and incubating the tubes for 60 minutes at room temperature for 60 minutes inbefore ova, sperm and larvae were used for fertilization (see Chapter 3: Target tissue) and larvae transferred to Erlenmeyer flask. All developing embryos and larvae were incubated at 18ºC for 3 days.

Initially, both ExGen 500 and the 25kDa PEI (prepared as previously described, see Chapter 3: Cytotoxicity) was used for transfection of sperm, ova and larvae. Due to the great number of cells that needed to be transfected and the high volumes of transfection reagent therefore necessary it was decided to utilize the 25kDa PEI for the majority of the transfection optimization. Using the 25kDa PEI was much more economically viable seeing that 500ml of undiluted 25kDa PEI costs 10X less than 1ml of ExGen 500. Transfections were carried out with the pTracer-CMV2 and pCMV-DsRed-Express constructs in their circular and linearized forms.

Fertilizations were performed with treated sperm (see Table 5.1) and untreated ova; untreated sperm and treated ova (see Table 5.2); and with treated sperm and treated ova. In cases where both sperm and ova were treated, sperm ($5.0x10^7$ cells/ml) was treated with the same concentration of transfection reagent and DNA as the ova were treated with (see Table 5.2).

Table 5.1: One millilitre sperm ($5.0x10^7$ cells/ml) treated with a DNA and 25kDa PEI concentration series. The combination and ratio of the DNA/PEI complex are indicated. Treatment was conducted in a 2ml eppendorf tube and incubated at room temperature for 30 minutes before sperm was used for fertilizations.

25kDa PEI (µg) \ DNA (µg)	1.00	3.00	5.00	10.00	50.00	150.00	250.00
1.00	(1:1)						
5.00	(1:5)		(1:1)				
10.00				(1:1)			
15.00		(1:5)	(1:5)				
50.00				(1:5)	(1:1)		(5:1)
150.00						(1:1)	
250.00					(1:5)		(1:1)

Table 5.2: One millilitre ova at a concentration of $1.0x10^3$/ml were treated with DNA and 25kDa PEI concentration series. The combination and ratio of the DNA/PEI complex is indicated. Treatment was conducted in a 2ml eppendorf tube and incubated at room temperature for 30 minutes before ova were used for fertilizations.

25kDa PEI (µg) \ DNA (µg)	1.0	1.50	3.00	10.00	50.00
1.50			(2:1)		
5.00	(1:5)				
7.50		(1:5)			
10.00				(1:1)	
15.00			(1:5)		
50.00				(1:5)	(1:1)
250.00				(1:25)	(1:5)

Transfections were also performed on larvae at a concentration range similar to that seen in Table 5.2. However as larvae consist of multiple cells, are less penetrative and are more resilient to transfection treatment, 80 larvae/ml was used instead of the $1.0x10^3$ larvae/ml

used in the previous experiment. This procedure mirrored a more than ten-fold increase in reagents concentration in comparison with the concentration of reagents ova were treated with. By decreasing the number of larvae that were treated instead of increasing the reagent concentration, the cost of reagents could be minimized. Treatment was conducted in a 2ml eppendorf tube and incubated at room temperature for 60 minutes before ova, sperm and larvae were transferred to 6-well plates containing 8ml FSW and incubated at 18ºC for 2 days before DNA was extracted (see Table 5.3).

Table 5.3: One millilitre of larvae (16 hours post-fertilization) at a concentration of 80 larvae/ml were treated with a DNA and 25kDa PEI concentration series. The combination and ratio of the DNA/PEI complex is indicated.

		DNA (µg)					
		1.00	2.00	3.00	5.00	6.00	10.00
25kDa PEI (µg)	10.00	(1:10)					
	15.00			(1:5)			
	20.00		(1:10)		(1:4)		(1:2)
	25.00						
	30.00					(1:5)	
	40.00						(1:4)

Due to cost factors the use of ExGen 500 was limited to treatment of the predicted target tissue that would render the best results; sperm. Sperm ($5.0x10^7$/ml) was treated with 1µg of DNA complexed with 2.75µl; 3.3µl; 3.84µl and 4.39µl of ExGen 500 as well as 10µg of plasmid DNA complexed with 17µl of ExGen 500.

All experiments were carried out using three biological replicates containing three technical replicates each.

5.2.3. Transfection validation

5.2.3.1. Microscopic analysis of transfected gametes, embryos and larvae

Gametes, embryos and larvae were fluorescently monitored using an Olympus IX51 fluorescent microscope with an EGFP (excitation: 450nm to 460nm; emission: 500nm to

550nm) and FITC (excitation: 460nm to 500nm; emission: 510nm to 560nm) filter. Larvae were monitored for a period of 2 to 7days (depending on larval survival under laboratory conditions).

However, due to autofluorescence exhibited by ova, embryos and larvae (see Chapter 2: Fluorescence), target tissues were subjected to PCR to confirm the presence of the EGFP gene (pTracer-CMV2) within the target cells. In order to simplify the validation procedure PCR was only carried out with primers for the EGFP gene and not for gene fragments contained in the pCMV-DsRed-Express construct as well. Larvae needed to be collected from Erlenmeyer flasks or 6-well plates and preserved until further validation studies could be completed. Larvae were therefore siphoned using 70µm nylon cell strainers (Lasec) for collection after at least 3 days of incubation. Larvae were washed from the strainer's surface using 99% (v/v) ethanol and stored in 1.5ml eppendorf tubes in 99% ethanol at 4ºC.

5.2.3.2. Removal of external plasmid DNA: DNase I trial

It was necessary to determine whether plasmids were internalized within target cells and therefore it was necessary to remove the external plasmid DNA before proceeding with DNA extraction and PCR. DNase I (Sigma-Aldrich) treatment was employed for this purpose. It was however necessary to assure that the DNase I treatment was sufficient in removing external plasmid DNA. A DNase I trial was therefore undertaken. Untransformed (untreated) larvae were collected and stored in 100% ethanol before samples were centrifuged and washed with autoclaved distilled water to remove ethanol. One µg of plasmid DNA was added to $1.0x10^3$ of these larvae. These samples were then treated with a concentration series (20.0µg/ml; 50.0µg/ml; 100.0µg/ml; 150.0µg/ml; 200µg/ml) of DNase I to determine the optimal concentration necessary for removal of external plasmid DNA. Controls that were treated with transfection reagent, but not plasmid DNA and controls that did not undergo either addition of plasmid DNA or DNase I treatment were also incorporated. Thereafter samples underwent PCR (see PCR section below) to assess the efficacy of the DNase I treatment. After the optimal DNase I concentration was determined, all samples that were subjected to transfection treatment underwent DNase I treatment as well. After DNase I treatment larvae samples underwent DNA extraction and PCR for validation of the presence of the transgene within the larval cells.

5.2.3.3. DNase I treatment and DNA extraction of transfected larvae

To remove ethanol and residual plasmid DNA, samples were pulse centrifuged and washed with autoclaved distilled autoclaved water. Some residual water was left in the tube and used to resuspend the larvae in. To ensure that all residual plasmid DNA was removed from the outside of the larvae, 200µg (found to be the optimal concentration during the DNAse I trail, see aforementioned section) of DNase I was added to the larval samples and incubated at 37℃ to facilitate the destruction of all exterior DNA. DNase I was inactivated by denaturation at 95℃ for 10 minutes. DNA was extracted using a SDS protocol [Aljanabi and Martinez 1997] and resuspended in autoclaved distilled water. The concentrations of these samples were examined by making use of a Nanodrop 1000 Spectrophotometer V3.6 (Thermo Fisher Scientific) before these samples were used in PCR.

5.2.3.4. PCR

Two separate PCR reactions were carried out on each larval sample. Samples underwent PCR with primers that amplify a 200bp fragment of the sperm lysin gene [Sweijd *et al*. 1998] that is located in the genome to confirm the presence of genomic abalone DNA. The primer sequences were as follows: 5'-CGTTCTAGAGAAACTTGATCCCTTCC-3' and 5'-GCGCTGCAGAAAAATATTTATTTAC-3'. A PCR cycle of 95℃ for 5 minutes followed by 35 cycles of 94℃ for 1 minute, 53℃ for 1 minute, 72° C for 30 seconds and then 72℃ for 2 minutes was followed.

The second PCR was carried out using primers specific for the EGFP gene in an attempt to confirm the presence of the pTracer-CMV2 plasmid. The EGFP primers and PCR conditions are similar to those found in Wang *et al*. (2004). The primers were as follows: 5'-CCAACACTTGTCACTACTTT-3' and 5'-GCTTTGATTCCATTCTTT-3' and amplify a central 300bp segment of the EGFP gene with a general PCR cycle of 95℃ for 60 seconds; 15 seconds at 95℃, 15 seconds at 55℃, 30 seconds at 72℃ for 35 cycles and 7 minutes at 72℃. Samples underwent agarose gel electrophoresis on a 2% (w/v) agarose gel in 1X TBE electrophoresis buffer containing 0.05mg/ml Ethidium Bromide to determine whether the PCR reaction had amplified the 270bp sperm lysin gene fragment as well as the 300bp EGFP fragment. Samples were visualized using the MultiGenius BioImaging System (Syngene).

5.2.4. Transfection of haemocytes

Haemocytes were collected and seeded at 5 to 8x10^4 haemocytes per well in a 96-well plate as previously described (see Chapter 3: Target tissue). Haemocytes were transfected either before seeding, on the day of seeding (with the addition of culture medium) or the day thereafter (24 hours after seeding). Transfection reagent and DNA complexes were prepared according to the manufacturer's protocol with the exception of one round of transfections where DNA and transfection reagent was added separately to cells to determine the effect on haemocyte transfection. Haemocytes were transfected with the pTracer-CMV2 and pCMV-DsRed-Express constructs in their circular and linearized forms (see Table 5.4).

Table 5.4: Haemocytes were treated with a series of either ExGen 500 or 25kDa PEI (5µg/ml) and also a series of DNA concentrations. The amount of DNA corresponding to the volume of ExGen 500 recommended by the manufacturer is indicated by an 'R'. All the combinations contained in the table were however tested.

		DNA (µg)						
		0.30	0.50	0.80	1.00	2.00	3.00	6.00
ExGen 500 /25kDa PEI (5µg/ml) (µl)	1.00	R						
	1.32	R						
	1.48		R					
	1.65		R					
	2.65			R				
	3.30				R			
	6.60					R		
	9.87						R	
	16.45							R

Further transfection experiments were carried out with haemocytes and these cells underwent flow cytometry analysis (see below). These haemocytes were treated as previously described with DNA/PEI complexes at a ratio of 1:10; 1:5; 1:2 with 0.3µg DNA: 3ug PEI; 0.5µg DNA: 2.5µg PEI and 1µg DNA: 2µg PEI respectively.

All experiments were carried out using three biological replicates containing three technical replicates each.

5.2.4.1. Microscopic analysis and flow cytometry of haemocytes

Haemocytes were fluorescently monitored with an Olympus IX51 fluorescent microscope with an EGFP (excitation: 450nm to 460nm; emission: 500nm to 550nm) and FITC (excitation: 460-500nm; emission: 510 to 560nm) filter for at least 7 days after transfection.

Haemocytes that were intended for flow cytometry analysis were harvested from cell cultures 3 days post-transfection to undergo flow cytometry analysis. Cells were harvested by scraping them from the culture plates with a sterilized self-fashioned glass bar and by pipetting vigorously. Loosened cells and culture medium was transferred to eppendorf tubes and centrifuged at low revolutions (3500g) for 1 minute before the supernatant was removed and replaced with Hank's balanced salt medium (Sigma-Aldrich). This was done 3 times to remove the coloured culture medium which could influence flow cytometry result and replaced with the transparent salt solution. Samples were analysed at the Central Analytical Facility at Stellenbosch University with a BD FACSAria flow cytometer. Flow cytometry results were analysed using FACSDiva version 6.1.2 software (BD Biosciences).

5.2.5. Transfection reagent validation

To validate the effectiveness of transfection reagents and plasmid DNA, mammalian (HepG2) cells were transfected with the same reagents used in transfection of target tissues. Hep2G cells were seeded at $1.0x10^5$/well in a 6-well plate and transfected after one day of incubation with one of the following; 3µg of either plasmids complexed with 9,87µl ExGen 500 or 15µg 25kDa PEI; 3µg plasmid complexed with 25µg PEI; and 0.5µg plasmid complexed with 1.65µl ExGen 500. Cells were incubated for 2 days before being investigated using an Olympus

IX51 fluorescent microscope with an EGFP (excitation: 450nm to 460nm; emission: 500nm to 550nm) and FITC (excitation: 460nm to 500nm; emission: 510nm to 560nm) filter.

Another transfection reagent, GeneJuice (Novagen) (a polyamine) was also used to transfect abalone haemocytes in a transfection trial with the pTracer-CMV2 and pCMV-DsRed-Express constructs. GeneJuice was prepared and used according to the manufacturer's specifications. Haemocytes were collected and seeded at 5 to 8x10^4 haemocytes per well in a 96-well plate as previously described (see Chapter 3: Target tissue) and transfected within 1 day of seeding (according to manufacturer's specifications) and treated with 80ng DNA: 0.2µl GeneJuice (1:5) or 160ng DNA: 0.4µl GeneJuice (1:2.5) as recommended by manufacturer's specifications. Cells were incubated for 2 days before being investigated using an Olympus IX51 fluorescent microscope with an EGFP (excitation: 450nm to 460nm; emission: 500nm to 550nm) and FITC (excitation: 460nm to 500nm; emission: 510nm to 560nm) filter.

5.3. Results

5.3.1. Plasmids

E. coli cells were successfully transfected with the pTracer-CMV2 construct and were observed to express the EGFP gene (see Figure 5.2)

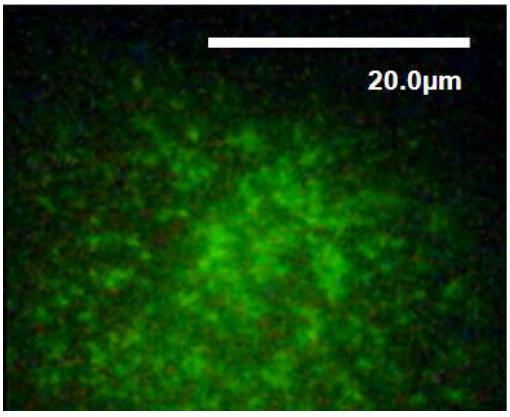

Figure 5.2: Transformed *E. coli* cultures expressed the EGFP protein contained in the pTracer-CMV2 gene construct and exhibited a strong fluorescent signal when examined under 100 times magnification with an EGFP filter (also see Figure 2.1).

The successful digestion of most if not all plasmid DNA was confirmed by gel electrophoreses. Plasmids that underwent restriction enzyme digestion exhibited a single band representative of only one conformation species; the linearized form. Plasmids that did not undergo restriction enzyme digestion exhibited two conformational species; open circular and supercoiled circular (see Figure 5.3). The presence of three bands after gel electrophoresis would be indicative of incomplete digestion. All plasmids used in further transfection experiments were confirmed to only display one band after gel electrophoresis.

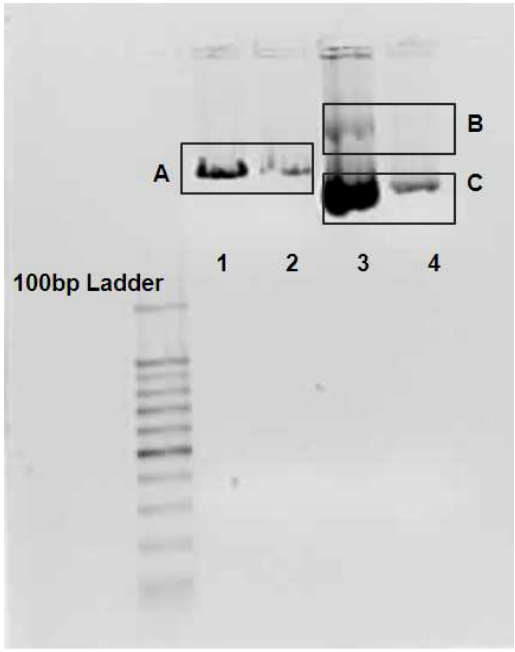

Figure 5.3: Samples containing 100ng (lane 1) and 10ng (lane 2) of the linearized 5.7kbp plasmid presented one band visible on the gel, while 1330ng (3) and 154ng of circular plasmid (4) presented with 2 bands. The single band was intermediate in size to the two bands observed for circular plasmids (1% denaturing agarose gel at 120V for 1 hour).

5.3.2. Transfection validation

5.3.2.1. Microscopic analysis of transfected gametes, embryos and larvae

Sperm that underwent transfection treatment did not exhibit any fluorescence within 30 minutes to an hour of incubation with the DNA/PEI complex before fertilization, nor within one day of transfection (if not used for fertilization). Due to the high degree of autofluorescence

the ova exhibit, expression of the fluorescent transgenes could not be examined. Larvae were examined for expression of the transfluorescent protein. Although the autofluorescent signal was still observed, it was presumed that expression of the transgene fluorescence would still be visible due to differential fluorescence, an increase in fluorescent intensity or an increase in the tissues exhibiting fluorescence. However, no difference in fluorescent intensity, distribution or colour compared to the control group could be observed for any of the transfection experiments.

5.3.2.2. Removal of external plasmid DNA: DNase I trial

DNase I concentrations of 50µg/ml; 20µg/ml and 0.0µg/ml were not sufficient to degrade 1µg of external plasmid DNA to such an extent to make PCR amplification of the target gene impossible. Concentrations of 200µg/ml; 150µg/ml and 100µg/ml however resulted in no visible PCR product and were therefore considered to degrade plasmid DNA sufficiently to eliminate the possibility of false positives (see Figure 5.4). To ensure the elimination of false positives, a concentration of 200µg/ml was used in further experiments.

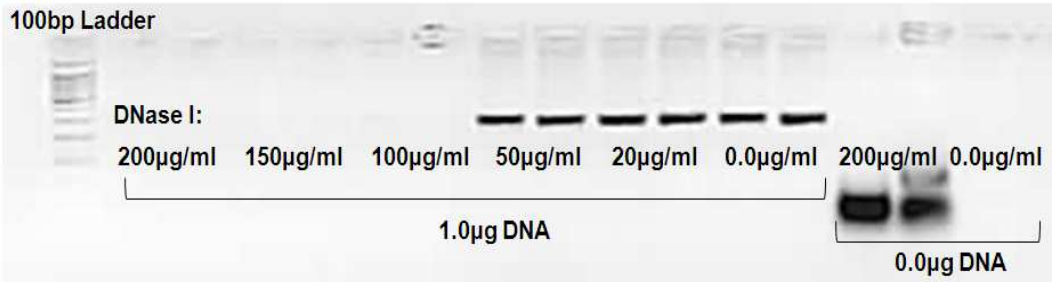

Figure 5.4: DNase I concentrations of 50µg/ml; 20µg/ml and 0.0µg/ml were insufficient to degrade external plasmid DNA to make the amplification of the 300bp EGFP fragment impossible. However, concentrations of 200µg/ml; 150µg/ml and 100µg/ml did not result in any amplifiable PCR substrate.

5.3.2.3. DNase I treatment, DNA extraction and PCR of transfected larvae

The practice of rinsing larvae with distilled autoclaved water followed by treatment with 200ug of DNase I per 1000 larvae was demonstrated to be effective in removing residual plasmid DNA (see Figure 5.5) while retaining the integrity of the larval structures to ensure efficient DNA extraction. Making use of PCR to confirm the effective extraction of genomic DNA by

amplification of a genomic gene, a fragment of the sperm lysin gene, and a plasmid gene fragment to confirm the presence of the pTracer-CMV2 construct was an efficient system to confirm the transfer of foreign DNA.

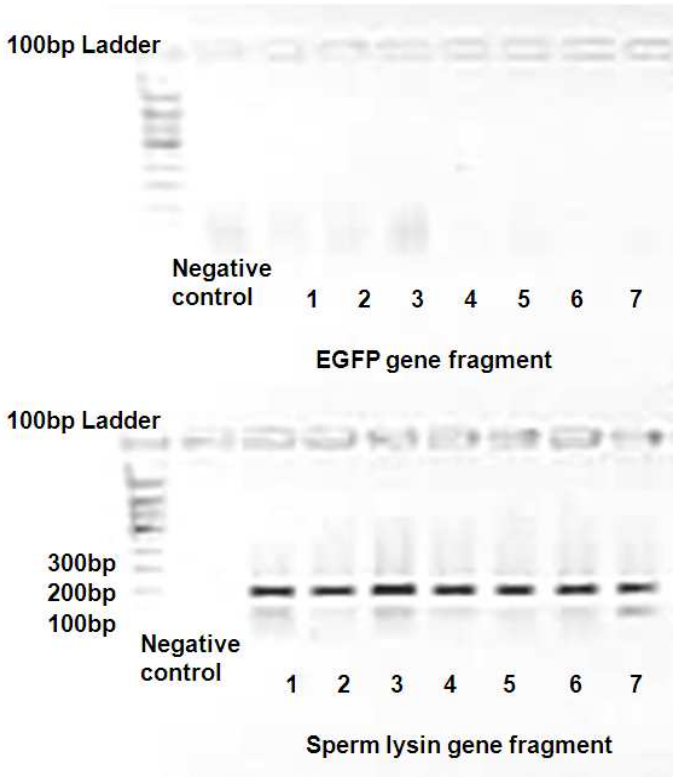

Figure 5.5: Samples 1-7 underwent PCR with the sperm lysin primers and the EGFP primers. In this case samples 1-7 did not contain the EGFP gene although satisfactory quality genomic DNA was extracted to yield apparent PCR products with the sperm lysin gene.

5.3.3. Transfection of gametes and larvae

Larval samples produced with sperm that was transfected prior to fertilization, exhibited the amplification of a 300bp fragment after PCR with the EGFP primers for some treatments (see Figure 5.6). From Figure 5.6 and 5.7 it is apparent that treating $5.0x10^7$ sperm cells with less than 10µg of DNA or less than 10µg 25kDa PEI was not successful in transferring the plasmid DNA to the larvae. Treating ova and sperm with the same amount of DNA and PEI yielded positive results when more than 10µg of DNA and PEI was added. A DNA: PEI ratio of 1:1 or 1:5 was most successful. Treating larvae and the exclusive treatment of ova was not

successful in producing larvae containing the transgenes. Although ExGen 500 was not experimented with extensively, 17µl of ExGen 500 with 10µg of DNA was successful in transferring the transgene to larvae after treatment of $5.0x10^7$ sperm. The configuration (circular or linearized) of the plasmid did not influence the success of transfection and yielded identical results.

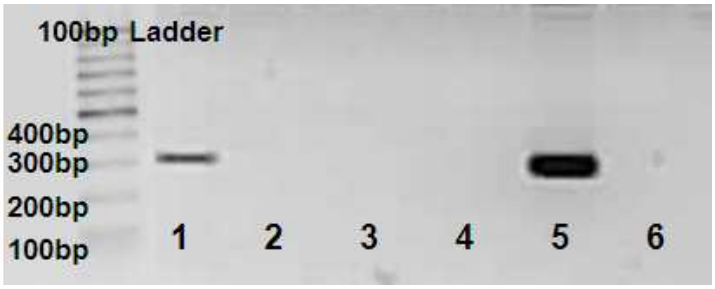

Figure 5.6: Sperm transfected with 10µg DNA/10µg 25kDa PEI and used in fertilizations resulted in larvae that were positive for the presence of the 300bp EGFP gene fragment (Lane 1). Lane 2-4 contained sperm treated with: 5µg DNA/5µg 25kDa PEI; 5µg DNA/15µg 25kDa PEI; 1µg DNA/1µg 25kDa PEI and 1µg DNA/5µg 25kDa PEI; all of which did not display the 300bp fragment. Lane 5 is a positive control and Lane 6 is a negative control.

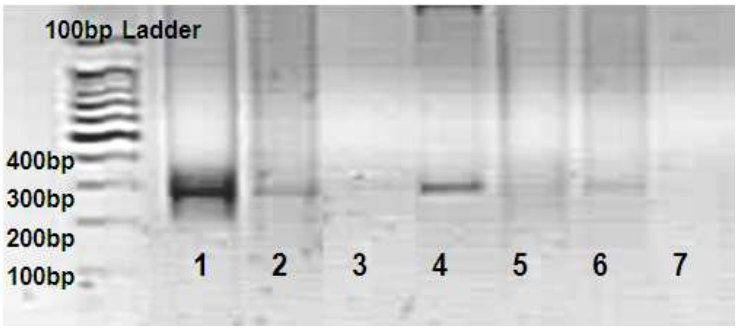

Figure 5.7: Treatment of $5.0x10^7$ sperm with 10µg DNA/17µl ExGen 500 resulted in larvae that contained the EGFP gene (Lane 1). Ova and sperm treated with 50µg DNA/50µg 25kDa PEI (Lane 2); 10µg DNA/10µg 25kDa PEI (Lane 3); 10µg DNA/50µg 25kDa PEI (Lane 4) and 3µg DNA/15µg 25kDa PEI (Lane 5). Only treatment with more than 10µg DNA/10µg 25kDa PEI resulted in a visible 300bp fragment. Lane 6 is a positive control and Lane 7, a negative control.

5.3.4. Transfection of haemocytes

5.3.4.1. Microscopic analysis and flow cytometry of haemocytes

The expression of the transgenes could not be confirmed either from fluorescent microscopic or flow cytometry analysis (see Figure 5.8 and 5.9). None of the concentrations of DNA, PEI or combinations thereof, different transfection reagents (ExGen 500, 25kDa PEI or GeneJuice), different times of transfection or the separate addition of PEI and DNA yielded cells that could be positively identified as expressing the transgenes.

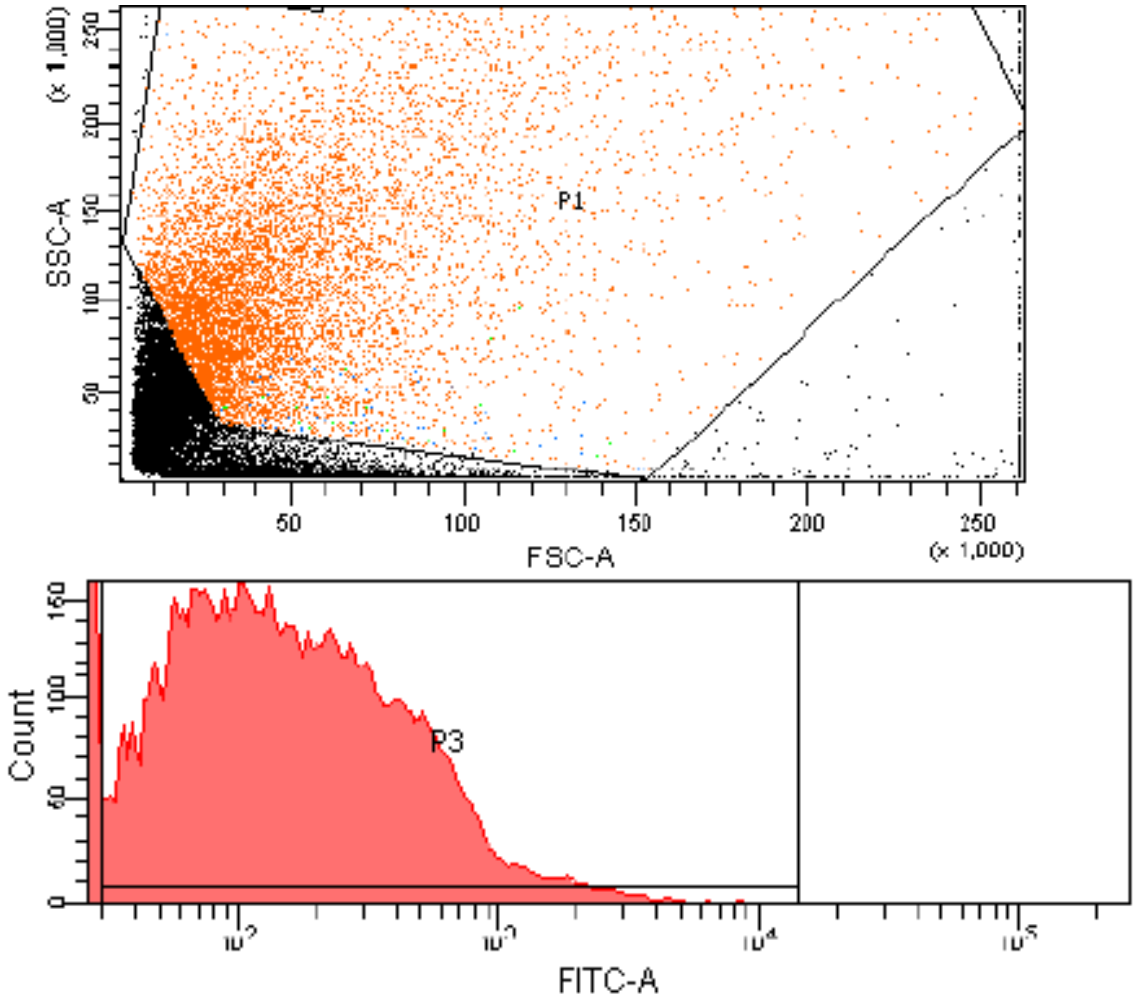

Figure 5.8: Flow cytometry results contained in a scatter plot and flow cytometric histogram indicated control sample of untreated cultured haemocyte cells to exhibit a fluorescently homogenous population of cells.

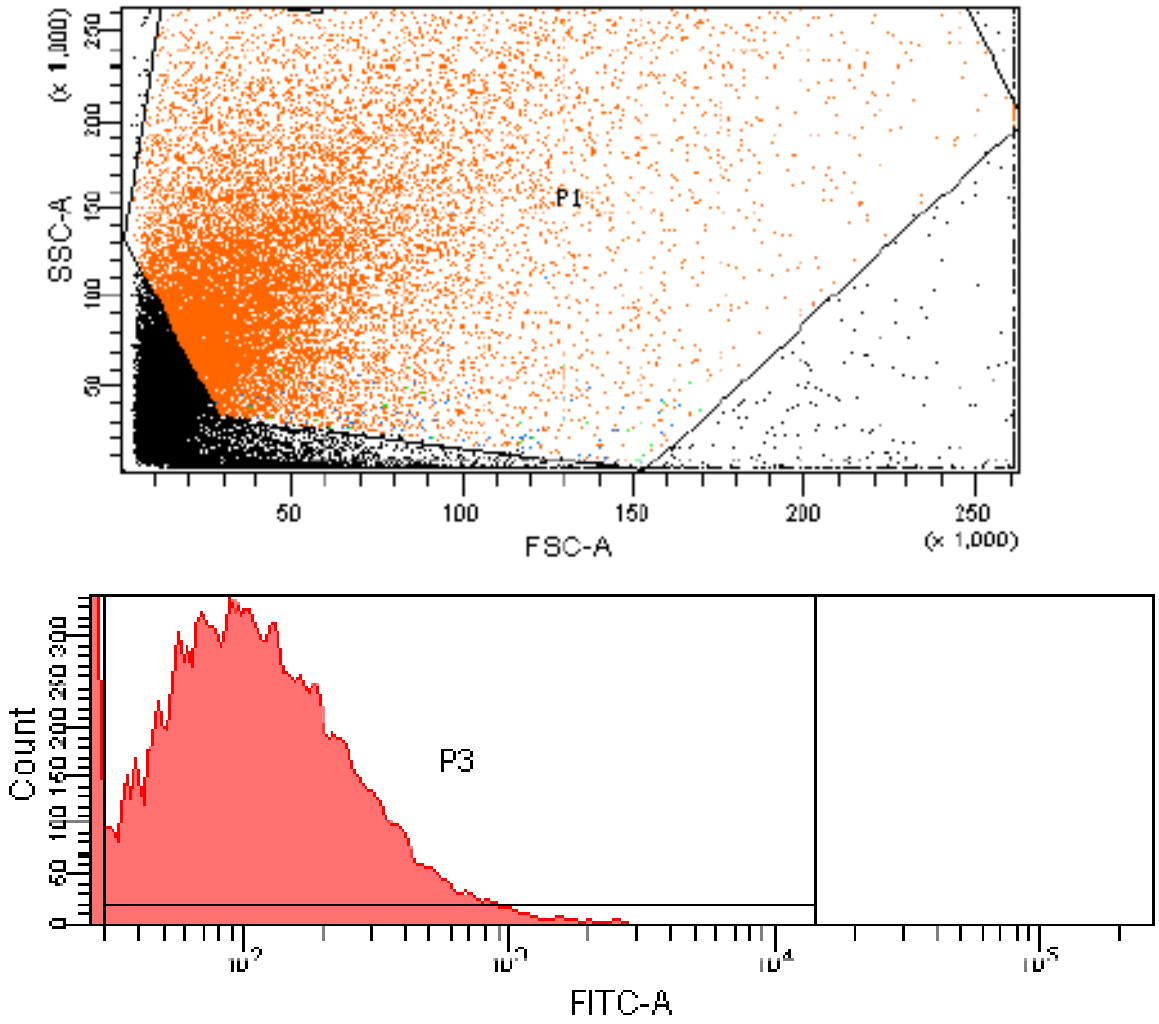

Figure 5.9: Treated haemocyte cells also exhibited a homogenous population of cells with regard to fluorescence with no significantly different levels of fluorescence as seen in this scatter plot and flow cytometric histogram.

5.3.5. Transfection reagent validation

Mammalian cells transfected with the pTracer-CMV2 and pCMV-DsRed-Express constructs and ExGen 500 and 25kDa PEI exhibited expression of the fluorescent proteins. Cells transfected with the pTracer-CMV2 (EGFP) construct exhibited green fluorescence, while cells transfected with the pCMV-DsRed-Express constructs exhibited red fluorescence regardless of the transfection reagent used (see Figure 5.10). Where cells were transfected with only 0.5µg DNA and 1.65µl ExGen 500, an obvious decrease in the amount of transfected cells could be seen.

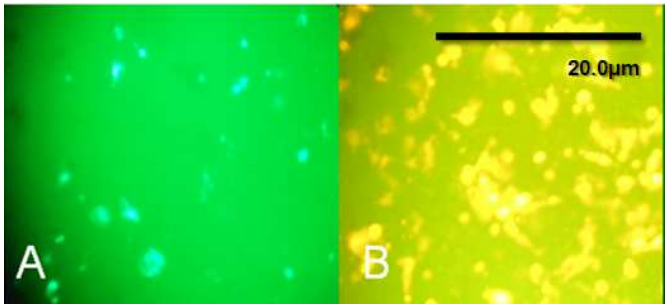

Figure 5.10: Untransformed Hep2G did not fluorescence under EGFP or FITC filters. Cells transformed by GeneJuice using EGFP (A) and DsRed (B) however fluoresced bright green and red respectively when viewed under EGFP and FITC filters.

The haemocytes treated with the GeneJuice transfection reagent did not exhibit fluorescence that could be distinguished by fluorescent microscopy investigation.

5.4. Discussion

5.4.1. Plasmids

Transformed *E. coli* cells were successfully transformed and expressed the transgenes that could be seen by the strong fluorescent signal produced. From these cells purified plasmid DNA of a high quality could be extracted. By making use of restriction enzymes with only an individual cut-site on a plasmid, the pTracer-CMV2 (EGFP) and pCMV-DsRed-Express (Ds-Red) were successfully linearized. The complete digestion of plasmids was successfully confirmed by gel electrophoresis; where one instead of two conformational species was present after complete digestion of the plasmids.

5.4.2. Transfection validation

5.4.2.1. Microscopic analysis of transfected gametes, embryos and larvae

No difference in fluorescent intensity, distribution or colour compared to the control group could be observed for any of the transfection treatments carried out on the gametes and larvae. Although it was presumed that larvae expressing the fluorescent transgenes would exhibit differential fluorescent intensity, distribution or colour, no such differences could be identified. It can therefore be concluded that none of the larvae expressed the fluorescent transgenes or that the expression of these genes did not alter or increase fluorescence in

such a way to make it obvious under fluorescent microscopy examination. Fortunately the presence of the transgenes within the larvae could be validated using PCR.

5.4.2.2. Removal of external plasmid DNA: DNase I trial

A DNase I trial indicated concentrations of less than 100µg/ml DNase I to be insufficient in degrading 1µg of external plasmid DNA. Although DNase I at a concentration of 100µg/ml degraded 1µg of DNA sufficiently to make PCR amplification of the EGFP transgene impossible, a concentration of 200µg/ml was used in further experiments. A generous amount of external plasmid DNA should be removed by the wash steps before reaching the DNase I step, however due to the fact that gametes and larvae would be treated with up to 250µg of plasmid DNA it was necessary to ensure that external plasmid DNA was completely degraded and did not results in false positives; thereby warranting the use of DNAse I at a concentration of 200µg/ml.

5.4.2.3. DNase I treatment, DNA extraction and PCR of transfected larvae

It was however also important to regulate the concentration of DNase I used due to the possibility of high enzyme concentrations resulting in the degradation of larval structures with the associated degradation of internal and genomic DNA. The use of a genomic gene (sperm lysin) that could be amplified with PCR and used to confirm the presence of intact genomic DNA was therefore necessary to prevent false negatives attributed to DNA degradation. Sperm lysin served to confirm the presence and integrity of genomic DNA and was shown to be an accurate indicator. Samples where the EGFP fragment was not amplified, was only noted as being unsuccessfully transfected when the sperm lysin gene fragment could be concurrently amplified.

5.4.3. Transfection of gametes and larvae

Sperm, ova, both sperm and ova, and larvae were treated with a series of DNA and 25kDa PEI. The DNA and 25kDa PEI series ranged from 1µg of DNA to 250µg, with the DNA/25kDa PEI ratio ranging from 1:1 to 1:25. The presence of the EGFP gene fragment was confirmed in larvae produced by sperm that were treated more than 10µg DNA and 10µg PEI as well as 10µg DNA/17µl ExGen 500. Sperm and ova treated with the same amount of DNA and transfection reagent also yielded larvae that contained the transgene. All treatments that

resulted in the successful transfer of the transgene to the larvae had a DNA/PEI ratio of 1:1or 1:5.

The amount and ratio of DNA and transfection reagent used for the treatments where both sperm and ova were transfected, were similar to those observed for treatment where only sperm was treated. The fact that transfection of ova exclusively did not result in the transfer of the transgene to the larvae indicates that the sperm are most probably responsible for the transfer of the transgene to the larvae in the experiments where both sperm and ova were treated. The inefficiency of transfer of transgenes from ova to larvae is most probably due to the fact that foreign DNA has difficulty penetrating the ova. The vitelline layer that envelops the mollusc ova have been shown to obstruct the transfer of foreign DNA to the ova [Esponda 2005].

Although the experimentation with ExGen 500 was limited, the instances where it was employed resulted in the transfer of the transgene to larvae after treatment of sperm with 10µg DNA/17µl ExGen 500. As previously stated 10µg of DNA seems to be necessary to treat $5.0x10^7$ sperm to result in transfer of the transgene to the larvae. Also it would seem that 17µl ExGen 500 or more would be necessary and that the 2.75µl to 4.39µl of ExGen 500 used in the trials in this study were insufficient.

5.4.4. Transfection of haemocytes

5.4.4.1. Microscopic analysis and flow cytometry of haemocytes

Haemocytes expressing either of the plasmid gene constructs could not be observed with fluorescent microscopy nor could a significant number of cells exhibiting significant fluorescence be identified using flow cytometric analysis.

ExGen 500 and the 25kDa PEI have been used successfully in several cultured tissue types [Morcos 2001; Erhardt *et al*. 2006; Von Gersdorff *et al.* 2006] and as in this study, for the transfer of transgenes to larvae by sperm. Also, ExGen 500 and the 25kDa were successfully used in conjunction with the pTracer-CMV2 (EGFP) and pCMV-DsRed-Express that were used in these trials, to transfect mammalian cells. It can be concluded that ExGen 500 and the 25kDa is most probably unable to transfect primary cultures of *H. midae* haemocytes and

that this is due to the inability of the cells to express the transgene. This is corroborated by the fact that the polyamine, GeneJuice, was also unable to transfect these cells.

Several authors have reported on the low level of proliferation and active cell cycling found in marine invertebrate cell culture. Auzoux-Bordenave *et al*. (2007) found only 8% of mantle cell cultures to be actively dividing. Lebel *et al.* (1996) stated that cell proliferation is a main concern with *in vitro* abalone (*H. tuberculata)* cell culture and that even tissues such as embryo or larval tissues exhibit low proliferation. A transfection study similar to the current study on marine invertebrate cell culture was proposed by Buolo *et al*. (2002); the transfection of cultured Pacific oyster heart tissue. After it was established that less than 5% of heart cells were exhibiting mitotic activity, compared to at least 50% exhibited by embryo cultures, the transfection of heart cells was abandoned. The low levels of proliferation and static cell cycling are most probably to blame for the lack of expression of the transgenes in this study.

5.4.5. Transfection reagent validation

The two transfection reagents, ExGen 500 and the 25kDa PEI, easily and successfully transfected mammalian cells using the manufacturer's protocol (ExGen 500) and the pTracer-CMV2 (EGFP) and pCMV-DsRed-Express constructs. This indicates the viability of these transfection reagents and plasmids for use in transfection studies.

The polyamine, GeneJuice was employed to transfect the haemocyte cell culture according to the manufacturer's specifications and using the pTracer-CMV2 (EGFP) and pCMV-DsRed-Express constructs. No cells expressing the transgene could be identified with fluorescent microscopy. A general difficulty in transfection on the part of the haemocytes can be assumed as none of the transfection reagents used could succeed in successful transfection and expression of the transgenes.

The pTracer-CMV2 (EGFP) gene-construct was delivered to the sperm cell by use of 10µg (or more) plasmid DNA complexed with 10µg (or more) 25kDa PEI. DNA verification by PCR can be utilized to test for the presence of the transgene. It was however not possible to determine the efficacy of transfections or to verify whether integration and expression of the construct had taken place. Future studies will benefit from a protocol that would enable PCR on

individual larvae to determine the transfection efficiency. Studies that maintain larvae for longer periods after transfection would be needed to confirm the integration and expression of the transgenes' expression analyses.

Haemocytes were not successfully transfected to express the transgenes most probably due to the lack of active proliferation and cell cycling. A proliferative cell line would most probably be a better candidate for transfection. Future studies could therefore focus on creation of such a cell line before attempting transfection.

It was verified that ExGen 500 and the 25kDa PEI can successfully act as transfection reagent for the transfer of plasmid DNA to *H. midae* larvae by using sperm as a vector. The future large-scale use of ExGen 500 is however doubtful due to the expensive nature of this transfection reagent. The 25kDa PEI offers a much more economical alternative.

Strict regulations regarding GMO housing, transport and disposal and the specialized nature of abalone rearing greatly confined the scope of this current study. The presence of the transgene in larvae could not be confirmed after more than a week due to the fact that possible GM larvae needed to be housed in a contained laboratory where they could not be kept viable for more than 7 days. The creation of a specialised contained laboratory containing facilities for settlement and grow-out of larvae as well as housing for spawning broodstock would be necessary to produce GM abalone beyond 7 days old.

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6. Putative growth genes

6.1. Introduction

6.1.1. Characterization of putative growth genes for future creation of a homologous gene construct

Assembly of a construct from genes, promoters and other regulatory elements originating from the transfection target's genome, a homologous gene construct, has become popular recently for transfection studies and has several advantages compared to the use of foreign, heterologous, gene constructs. Homologous gene constructs contain genes that are necessarily adapted to the species it is to be expressed in by being adapted to the organism's transcription regulatory and expression systems and therefore will most probably be expressed more efficiently [Gomez-Chiarri 1999]. Additionally, homologous transfection would in part calm consumer concern about food safety and ethical concern associated with the genetic modification [Nam *et al*. 2001]. Homologous gene constructs have been used to great success in some fish species, such as the mud loach (*Misgurnus mizolepis*) and several salmon species [Devlin *et al*. 1995; Nam *et al*. 2001].

The abalone genome has remained relatively unchartered with regard to pin-pointing genes and function. Although several abalone genes have been elucidated [Jackson and Degnan 2006], growth hormone genes have not yet been clearly characterized nor has the biological pathway responsible for growth been determined for haliotids. Putative genes involved in growth have only recently been identified for *Haliotis midae* [Van der Merwe 2010]. Consequently, an all-abalone gene construct does not exist and thus far abalone transfection studies have made use of heterologous gene constructs [Powers *et al*. 1995; Sin *et al*. 1995; Tsai *et al*. 1997, 2000; Wang *et al*. 2004].

Abalone undergo stages of varying growth measured by weight, shell length and netto production mass. Abalone growth (at least weight) is presumed to follow an exponential curve that tapers off as animals age [Reaburn and Edwards 2003; Park *et al*. 2008]. A reduction in growth rate in molluscs is often seen as animals reach sexual maturity and start spawning, partly due to energy allocation to gametogenesis instead of growth [Nell 2002]. These changes in growth potential are most probably due to differing expression profiles of genes involved in growth and development. By making use of RNA extraction, reverse transcription

followed by quantitative real-time PCR, these expression differences can be investigated in an attempt to characterize putative genes responsible for growth in *H. midae*. A previous study has identified genes that could possibly be involved with growth by comparison of expression profiles of exceptionally large (fast growers) and exceptionally small (slow growers) two year old full-sib individuals from a single spawning event [Van der Merwe 2010]. Three genes, preliminary named 752, 135 and 238, emerged as the most probable genes responsible for increased growth. All three these genes had increased expression of between 2.7 and 16.2 times in larger animals than smaller ones. These genes were annotated by making use of a BLAST (Basic Local Alignment Search Tool) algorithms to search various online databases. Gene 752 was identified to be an insulin related peptide receptor protein involved in several pathways (GenBank accession number: Q7YT64). Gene 13596 (referred to as 135) was identified as the perlustrin gene; a protein responsible for shell formation in molluscs (GenBank accession number: Q95V57); and gene 2380 (referred to as 238) was found to resemble a thrombospondin-like gene that is involved in, amongst other things, the inflammatory response (GenBank accession number: P35448). Genes that could serve as reference genes were also identified; being characterized by equal expression during expression analysis. To confirm the suitability of the genes as reference genes they were evaluated using the geNorm algorithm [Vandesompele *et al*. 2002] to assess their expression stability. These two genes 8629 (referred to as 862), a ribosomal protein S9 (GenBank accession number: Q7ZYU4); and 12621 (referred to as 126), an ornithine decarboxylase gene (GenBank accession number: P27117), were found to be suitable as reference genes according to the geNorm algorithm [Van der Merwe 2010].

6.1.2. Quantitative real-time reverse transcription PCR (qRT-PCR)

Quantitative real-time PCR is open to erroneous results due to a flawed experimental and quantitation procedures. To prevent erroneous results several guidelines for experimental and quantitation procedures need to be followed. Several authors [Pfaffl *et al*. 2002; Van Guilder *et al*. 2008; Bustin *et al*. 2009; Guénin *et al*. 2009; Derveaux *et al.* 2010; Taylor *et al*. 2010] have therefore highlighted some of the crucial elements to successful quantitative real-time PCR: Firstly, it is critical to ensure the integrity and purity of the initial RNA. RNA samples should be of high-quality, DNA free and undegraded to ensure accurate assessment of this starting RNA. Accuracy and precision should be ensured when carrying out qRT-PCR

reactions. Secondly, it is vital to ensure constant amplification efficiencies between all samples that are to be compared. The efficiency of samples greatly influences the accuracy of calculated expression results. Amplification efficiency between target samples and stably expressed reference genes therefore need to be invariable as far as possible. The use of quantification models that employ efficiency correction is also strongly recommended. The relative expression software tool (REST) [Pfaffl *et al*. 2002] employs a relative calculation procedure based on the mean crossing point (CP) of the target and reference:

$$\text{Expression ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{mean control-mean sample})}}{(E_{\text{target}})^{\Delta CP_{\text{reference}}(\text{mean control-mean sample})}}$$

REST also employs a randomization test with pair-wise reallocation, seen as the most appropriate statistical test for quantitative expression analysis, to indicate the significance of the difference between control and sample group (p-value).

Thirdly, normalization with more than one, preferably several stably expressed reference genes, is necessary to ensure accurate interpretation of data. Several algorithms, such as the geNorm method is available to assess a gene's suitability as a reference gene. The geNorm program ranks several genes according to relative expression data to indicate the most suitable reference gene for experimentation studies [Vandesompele *et al*. 2002].

Therefore in the current study, these guidelines were adhered to as closely as possible to establish whether genes identified as putative growth genes (genes 752, 135, 238) are differentially expressed and could possibly be responsible for growth differences between different life stages of *H. midae*. Three groups of abalone comprising 3 distinct life stages were incorporated in qRT-PCR analysis. The three groups were chosen to distinguish the effect of sexual maturation and spawning on growth as well as the overall difference in expression in the genes linked to growth. The groups were as follows; one year old juveniles; two to three year old that did not exhibit the onset of obvious gonad development or spawning and; four year old animals that had reached sexual maturity and were spawning without induction.

6.2. Materials and methods

6.2.1. Sampling

Five animals from the three groups; one year olds (Group 1); two to three year olds (Group 2) and; four year old that have commenced spawning (Group 3), were collected from a commercial abalone farm. The animals were randomly selected with the only prerequisites being that the animals were of the correct age, developmental stage and were in good health. In an attempt to minimise the stress experienced by the animals they were kept in their usual production housing until right before each individual was to be sacrificed. Animals were sacrificed on the premises in an enclosed laboratory to further minimise travelling and stress to the animals. Using a sterile scalpel, tissue (abductor muscle, mantle, gonadal tissue, intestinal tissue and dorsal ganglia) was harvested and cut into 125mm^3 sections and placed in RNAlater solution (Ambion) to preserve RNA. Samples contained in the RNAlater solution were incubated at 4ºC overnight to ensure the saturation of the tissues with the solution before incubation at -20ºC. Animals were measured and weighed before being sacrificed and an ANOVA was performed to confirm the significant difference in weight and length between groups. If a p-value of less than 0.05 was achieved, indicating there to be a significant difference between groups, a t-test was performed between paired groups for all the groups.

6.2.2. RNA extraction

All non-sterile and non-autoclavable plasticware, work surfaces and electrophoresis equipment was soaked overnight in a 0.1% SDS 0.1M NaOH solution and incubated at 37ºC before being rinsed with autoclaved milliQ water (Millipore Corporation). Glassware and stainless steel equipment was washed using a detergent, rinsed with autoclaved milliQ (Millipore Corporation) water and incubated overnight at 160ºC. Sterile, nuclease-free non-reusable plasticware was utilized with sterile pipettes used for RNA extractions.

From the harvested tissue samples, 2.5g of tissue comprising equal amounts of abductor muscle, mantle, gonadal tissue, intestinal tissue and dorsal ganglia were added to 50ml tubes containing 11ml extraction buffer (50mM Tris-HCl, 5mM $MgCl_2$, 150mM KCl, 10mM β-Mercapto-ethanol, 0.8M Sucrose). The tissue was homogenised using a polytron homogeniser (Brinkman). The homogeniser was cleaned between samples using 100% ethanol and RNaseZap solution (Ambion). Homogenised tissue was centrifuged at 15500g at

4ºC for 15 minutes and the supernatant transferred to a 50ml tube containing 3ml autoclaved milliQ water and an equal volume of SDS buffer (20mM Tris-HCL, 1% (w/v) SDS, 200mM NaCl, 40mM EDTA, 20ng/ml Proteinase K) and incubated at 45ºC for 40 minutes. An equal volume of a Phenol:Chloroform:Isoamyl alcohol (P:C:I) with a ratio of 50: 49: 1 was added and the sample was vortexed and placed on ice for 15 minutes for the phases to separate followed by centrifugation at 10000g at 20ºC for 10 minutes. The top liquid phase was then transferred to a new tube and an equal volume of P:C:I was once again added. This was repeated 3 times before 3ml of a 2M LiCl solution was added followed by an equal volume 2-propanol. The samples were incubated overnight at -20ºC before being centrifuged at 15000g at -20ºC for 60 minutes to form a pellet. The supernatant was discarded and 500µl of autoclaved milliQ water was added before the tube was vortexed to dissolve the pellet. The absorbance value of each sample at 230nm, 260nm and 280nm as well as the concentration of nucleic acids were recorded using a Nanodrop® ND-1000 Spectrophotometer. Samples were further purified using the RNeasy midi kit (Qiagen) according to the manufacturer's specifications followed by recording of the absorbance values of each sample in an attempt to determine whether the purification step was successful.

The integrity of samples was assessed by denaturing agarose gel electrophoresis. A 2% agarose gel was prepared containing 3% (w/v) formaldehyde and 1X MOPS buffer (see Appendix). A pre-run of 10 minutes at 50 volts was followed by electrophoresis at 50 volts for 90 minutes. Samples were prepared by incubation of 5µl of sample with 5µl of RNA loading dye at 65ºC for 20 minutes and incubation on ice for 2 minutes after which samples were loaded onto the gel.

Before reverse transcription of samples was undertaken, samples underwent a DNase digestion to remove any DNA that could result in inaccurate quantification in later steps. DNase digestion was carried out using 50µl of RNA sample, 5µl of 10X Turbo buffer and 1µl of Turbo DNase (Ambion) and incubated at 37ºC for 30 minutes before 5µl of the DNase inactivation reagent was added. After addition of the inactivation reagent, samples were incubated at room temperature for 5 minutes and then centrifuged at 10000g for 1.5 minutes before the supernatant was transferred to a new tube and the absorbance readings recorded.

6.2.3. Reverse transcription

To initiate reverse transcription, 800ng of RNA sample was added to 1µl of oligo(dT)$_{18}$ (100µM) primer (Fermentas), dNTPs (10mM) and 5µl of autoclaved milliQ water and incubated at 65ºC for 5 minutes and on ice for 1 minute. Thereafter 4µl of 5X First Strand buffer (Invitrogen), 1µl DTT (0.1M) (Invitrogen), 1µl Ribolock (40U/µl) (Fermentas) and 1µl of SuperScript III (Invitrogen) was added to the reaction, mixed by pipetting and incubated at 55ºC for 50 minutes and 70ºC for 19 minutes before undergoing RNAse H treatment. “No reverse transcriptase” controls were also incorporated. Remaining RNA, complementary to cDNA, was removed by using 1µl of RNase H (Fermentas) per 50µl of sample and incubated at 37ºC for 20 minutes, followed by inactivation at 65ºC for 10 minutes. Samples were frozen at -80ºC until further use.

6.2.4. Real-time PCR

Real-time PCR reactions were performed in 20µl reactions containing 1µl of sample, 10µl of SYBRFAST qPCR master mix (Kapa Biosystems), 0.4µl of both the forward and reverse primers (100µM) and 8.2µl of autoclaved milliQ water. All reactions were performed on the Corbett Rotor-Gene 6000 (Qiagen) using the 72-well rotor and with the appropriate Corbett real-time PCR tubes with a cycle of 95ºC for 5 minutes and then 40 cycles at 95ºC for 30 seconds, 58ºC for 30 seconds and 72ºC for 30 seconds. Primers were obtained from Mathilde van der Merwe [Van der Merwe 2010]; the primer sequences and product size is indicated in Table 6.1.

Table 6.1: Primers for qRT-PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
752	CTTCACTGGGGATTCGGAC	AGGATGCGAATGAGAAGAGTG	206
238	GACCAATCGCTCCATCCAC	GAGGACCCGCCAGTGTAAC	132
135	TTTATACGCTTCTATCTTGTTCCC	CCCTGTATTCTGATGCCTTGT	218
862	CATCCAACACACCAATACGC	TTGATGAGAAGGAACCCAGAC	78
126	TCATCGTCCCCAGTCACC	GCTTTCACTCTCACTGCCAAC	101

Standard curves were constructed to determine the accuracy of the assay, to establish the amplification efficiency and identify the most suitable dilution factor for further use. Pooled cDNA from all the samples used for quantification was utilized to construct the standard curves. The standard dilution series started at a concentration of 1200ng/µl followed by 5 other dilutions each a five-fold dilution from the previous standard.

The optimum dilution (1 in 25) could be gauged from the standard curves, the samples were all diluted by this factor and a suitable calibrator (pool of samples) at this dilution was incorporated into each run for each primer pair to ensure comparability between runs [Derveux *et al*. 2010]. Samples underwent real-time PCR with the primers for the three growth-linked genes and the two reference genes. A positive control (50ng cDNA), negative (no template) and “no reverse transcriptase control” (RNA) was included for each primer pair in each run. All real-time PCR reactions were done in triplicate.

6.2.5. Data analysis

Standard curves were constructed from data obtained in triplicate using Rotor-Gene 6000 version 1.7.87 software (Corbett Research) by making use of the *Quantitation* function followed by *Slope correct* and *Auto-find threshold* to ensure that the linear function created has the best fit.

The relative expression was determined by calculating the ratio between the gene expression of the gene of interest and the geometric mean of the two reference genes relative to the calibrator sample using REST 2009. This was done by generating crossing points and amplification efficiencies in Rotor-Gene 6000 version 1.7.87 software (Corbett Research) by applying the *Comparative quantification* analysis and exporting these values for each sample to the RG mode of REST 2009. Due to the nature of the data and the design of REST 2009, one of the groups was used as an *Untreated sample* from where the expression of the other group it was compared to was calculated. For each gene the three groups were compared pairwise; group 1 was compared to group 2, group 1 to 3; and group 2 to group 3, where the first group was then entered as an *Untreated sample*.

6.3. Results

6.3.1. Sampling

A significant difference (p-value < 0.05) between the length and weight of all three sample groups were indicated by the ANOVA and a t-test performed between paired groups (see Table 6.2).

Table 6.2: Sampled animal were given an arbitrary reference number and their weight and length recorded before being sacrificed.

Reference number	Length (cm)	Weight (g)
Group 1 : Animals of 1 year old and weighing at least 2.45g		
1.1	2.4	2.69
1.2	3.2	4.79
1.6	2.4	2.47
1.7	2.8	4.78
1.9	2.3	2.45
Group 2: Juvenile animals of 2 to 3 years old that have not spawned before and weigh at least 40g		
2.1	6.0	41.00
2.2	6.0	47.64
2.3	6.5	49.00
2.8	6.0	48.30
2.9	5.6	47.30
Group 3: Mature animals of 4 years old that have spawned before and weigh at least 90g		
3.1	8.0	105.60
3.5	7.5	92.80
3.7	7.8	91.50
3.8	7.5	91.80
3.9	8.0	102.74

6.3.2. RNA extraction

Before purification by the RNeasy midi kit (Qiagen) RNA concentrations were in the range of 2000ng/µl with the 260/280nm absorbance ratio being between 1.60 and 1.80 and the 260/230nm ratio being between 1.5 and 1.9. After purification the RNA concentration was in the range of 800ng/µl and the 260/280 ratio 2.05 and the 260/230 ratio 2.40. A 260/280 ratio of 2.0 and a 260/230 ratio of 2.0-2.2 is accepted as pure for RNA. Although the RNA

concentration decreased 2.5 fold, there was an appreciable shift in the 260/280 and 260/230 ratio towards values indicating higher purity [Taylor *et al*. 2010].

The RNA gel electrophoresis results revealed the samples to be intact with one clearly defined band visible (see Figure 6.1).

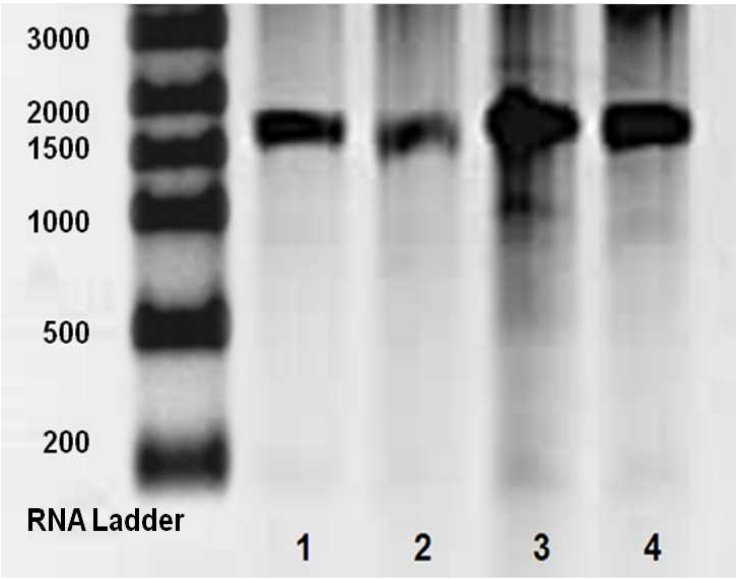

Figure 6.1: RNA isolated from whole abalone samples (Lanes 1 to 4) (2% denaturing agarose gel) with only one band indicating the 18S ribosomal subunit.

6.3.3. Quantitative RT-PCR

Standard curves indicated that reaction efficiencies for all genes to be between 98% and 108%. The r and r^2 values, indicating how well the regression line fits the data points, were more than 0.998 and 0.989 respectively (see Figure 6.2 and 6.3).

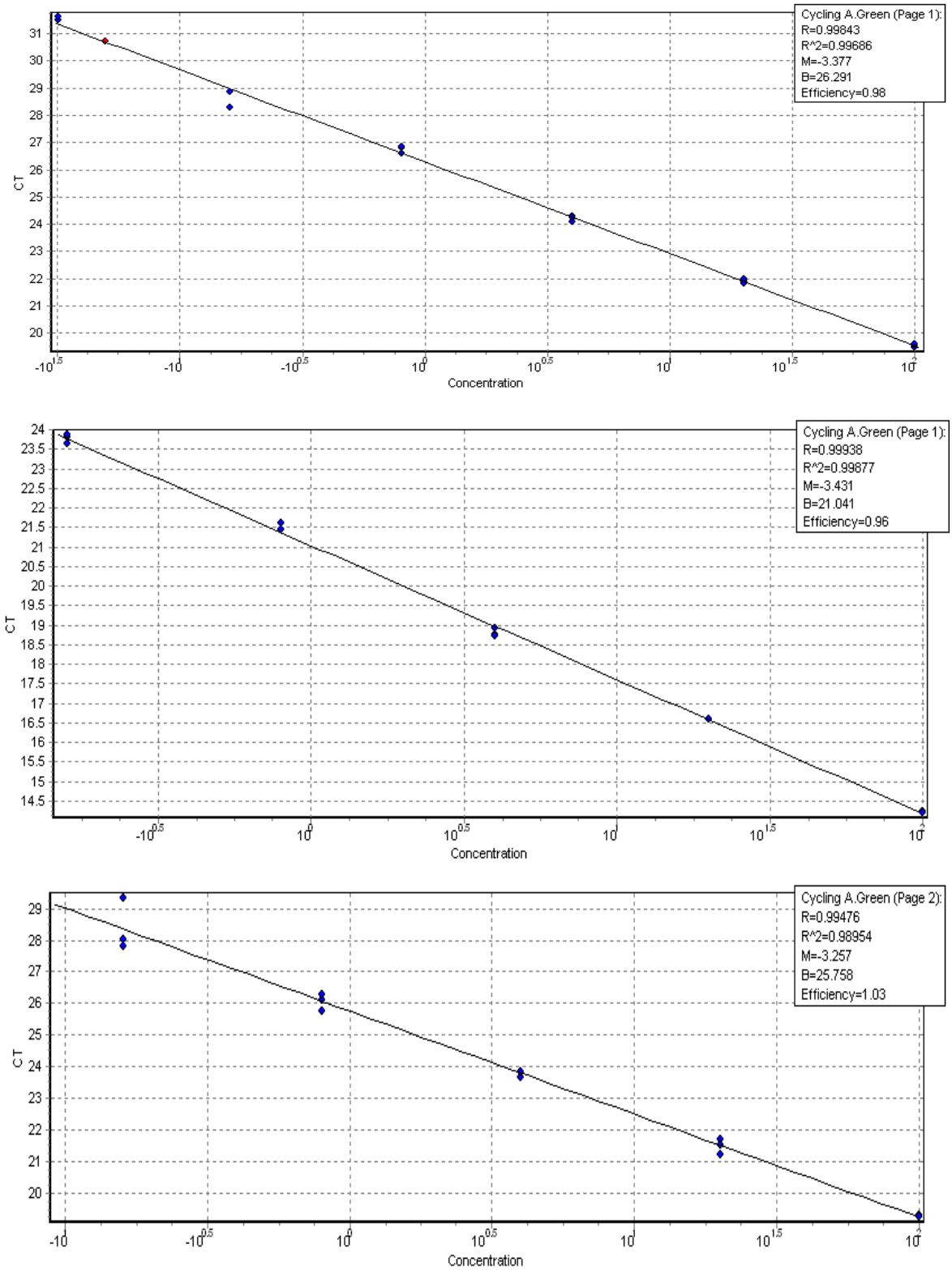

Figure 6.2: Standard curves of target genes 238, 135 and 752.

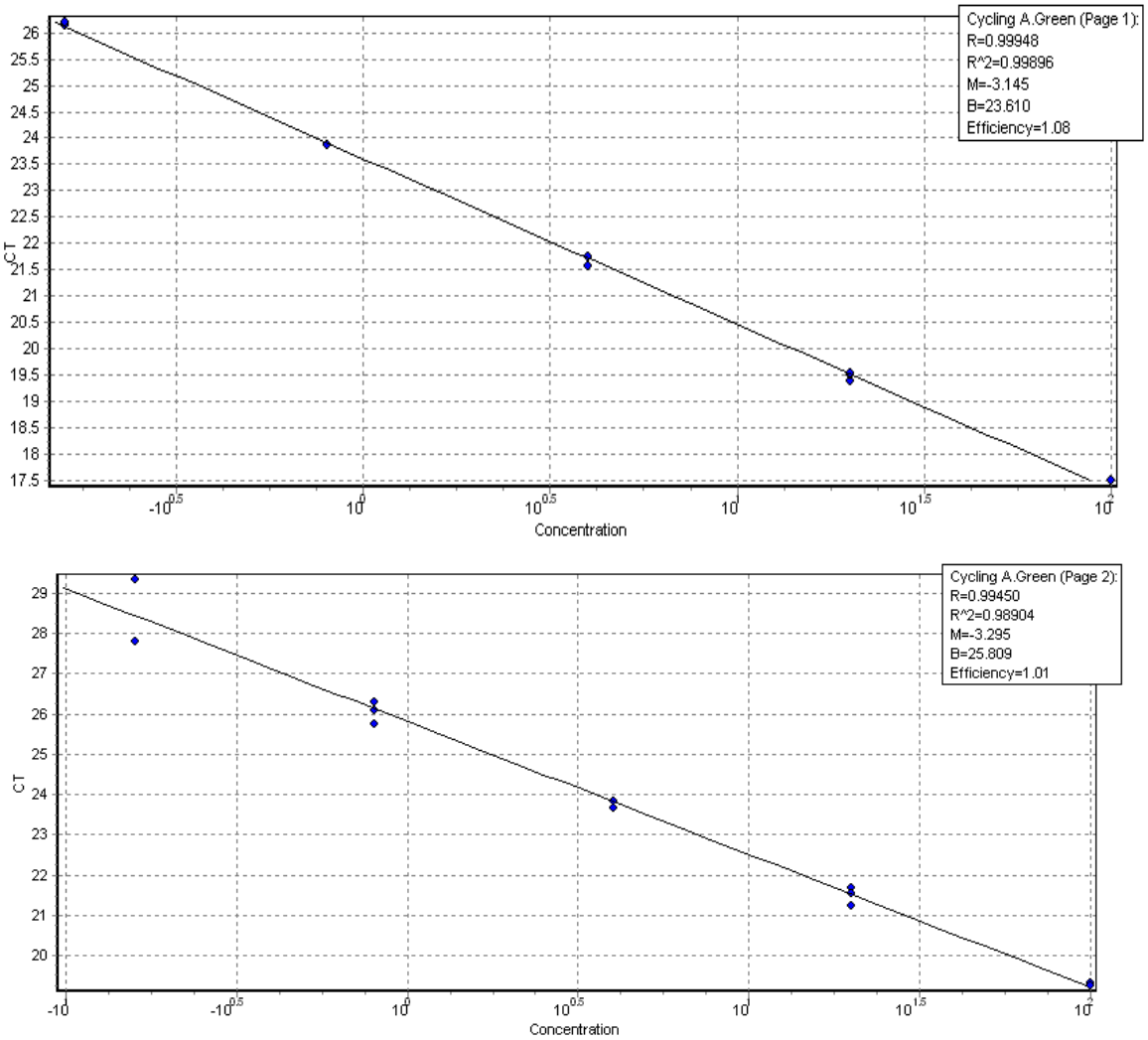

Figure 6.3: Standard curves for reference genes 126 and 862.

Melt curve analysis following real-time PCR indicated primer specificity and accuracy of the reaction procedure (Figure 6.4).

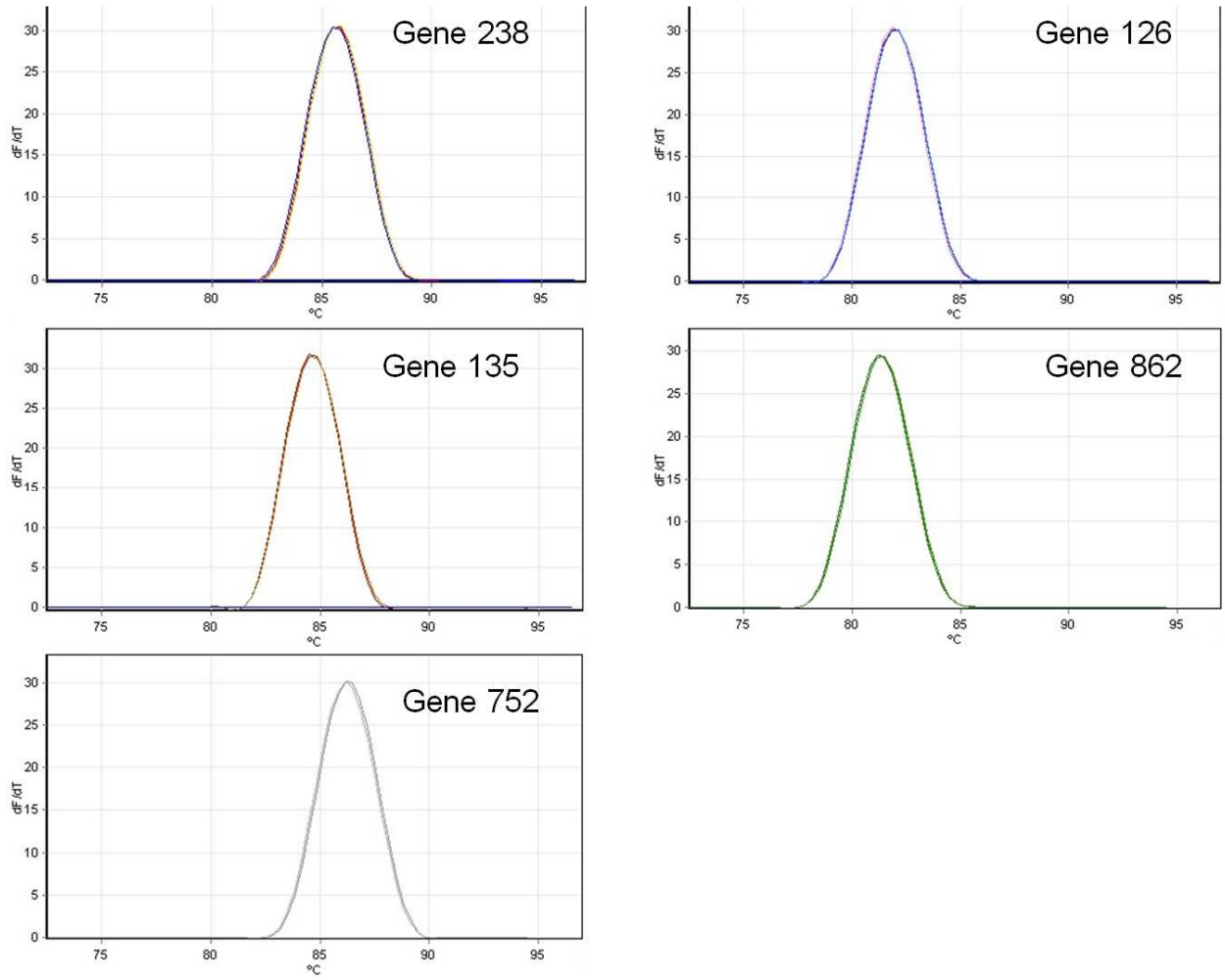

Figure 6.4: Melt curve analysis of the primer pairs of the five genes indicated amplification of the specific targets.

Table 6.3 and Figure 6.5 indicate that significant differences could not be detected when comparing expression of gene 238 in group1 to 2; group1 to 3 or group 2 to 3.

Table 6.3: REST 2009 results indicated there to be no significant difference in expression of gene 238.

Gene	Standard error	95% Confidence interval	p-value
238: Group 1 and 2	0.143 - 47.640	0.017 - 311.291	0.126
238: Group 1 and 3	0.033 - 4.395	0.001 - 11,323.403	0.386
238: Group 2 and 3	0.011 - 11.044	0.002 - 13,713.927	0.355

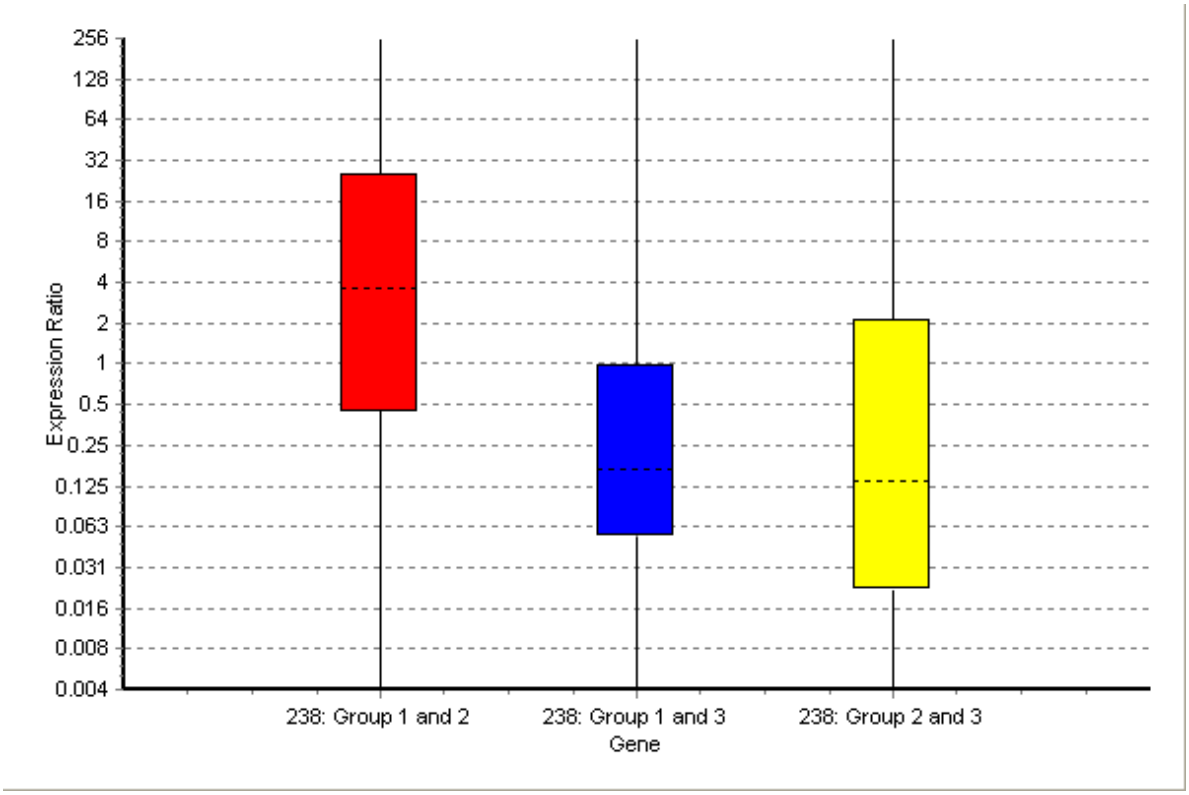

Figure 6.5: A box-and-whisker plot generated in REST 2009 for expression of gene 238, with dashed lines indicating the mean of each box-plot.

The REST 2009 results indicated there to be a significant difference in expression of gene 135 between all the groups (p-value < 0.05). Expression of gene 135 was up-regulated in group 2 in comparison with group 1; group 3 was up-regulated in comparison with group 1 and group 3 was up-regulated in comparison with group 2. The greatest difference in

expression was seen between group 1 and 3; group 3 had a 37.692 times higher mean expression of the gene of interest than group 1 (Table 6.4; Figure 6.6).

Table 6.4: REST 2009 detected significant differences in expression of gene 135 between all the groups.

Gene	Standard error	95% Confidence interval	p-value	Result	Fold difference
135: Group 1 and 2	0.224 - 178.028	0.073 - 9,615.354	0.016	UP	13.286
135: Group 1 and 3	2.415 - 826.941	0.413 - 28,171.507	0.000	UP	37.692
135: Group 2 and 3	0.893 - 165.396	0.179 - 3,050.118	0.002	UP	14.094

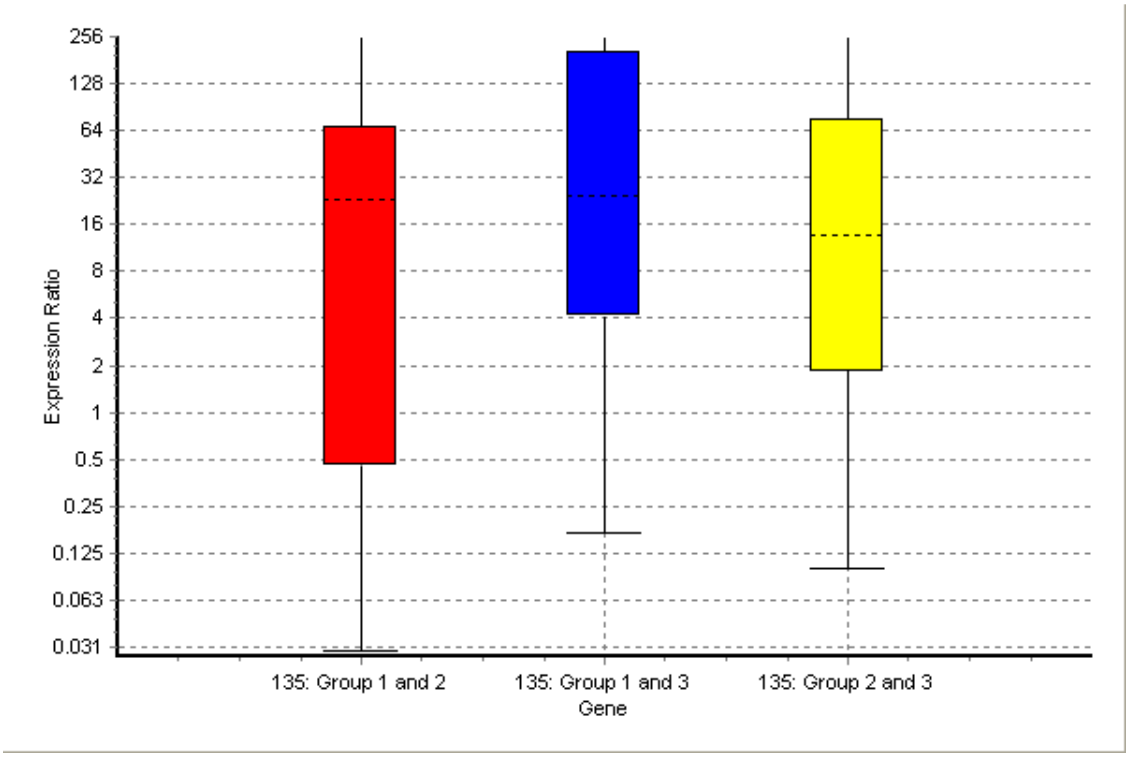

Figure 6.6: The box-and-whisker plot generated in REST 2009 for expression of gene 135, with dashed lines indicating the mean of each box-plot. All the groups that were compared differed significantly in expression.

The REST 2009 results indicated there to be significant differences in expression of gene 752 between the groups (p-value < 0.05). Expression of gene 752 was down-regulated in group 3 in comparison with group 1 and group 3 was down-regulated in comparison with group 2. The fold difference between group 1 and 3; and group 2 and 3 were in the same range (0.032 and 0.037) (Table 6.5; Figure 6.7). The reaction efficiencies, as determined by REST 2009, were between the recommended 90 to 110% [Taylor *et al*. 2010]. The box-and-whisker plot of all three genes indicated there to be a high degree of variability in the expression data (seen in the whiskers that represent the outer 50% of data) [Herrmann and Pfaffl 2005].

Table 6.5: REST 2009 results indicated there to be a significant difference in expression when comparing groups 1 and 3; and 2 and 3 for gene 752.

Gene	Standard error	95% Confidence interval	p-value	Result	Fold difference
752: Group 1 and 2	0.137 - 6.218	0.037 - 105.281	0.730		
752: Group 1 and 3	0.006 - 0.137	0.003 - 2.085	0.000	DOWN	0.032
752: Group 2 and 3	0.004 - 0.358	0.001 - 1.966	0.000	DOWN	0.037

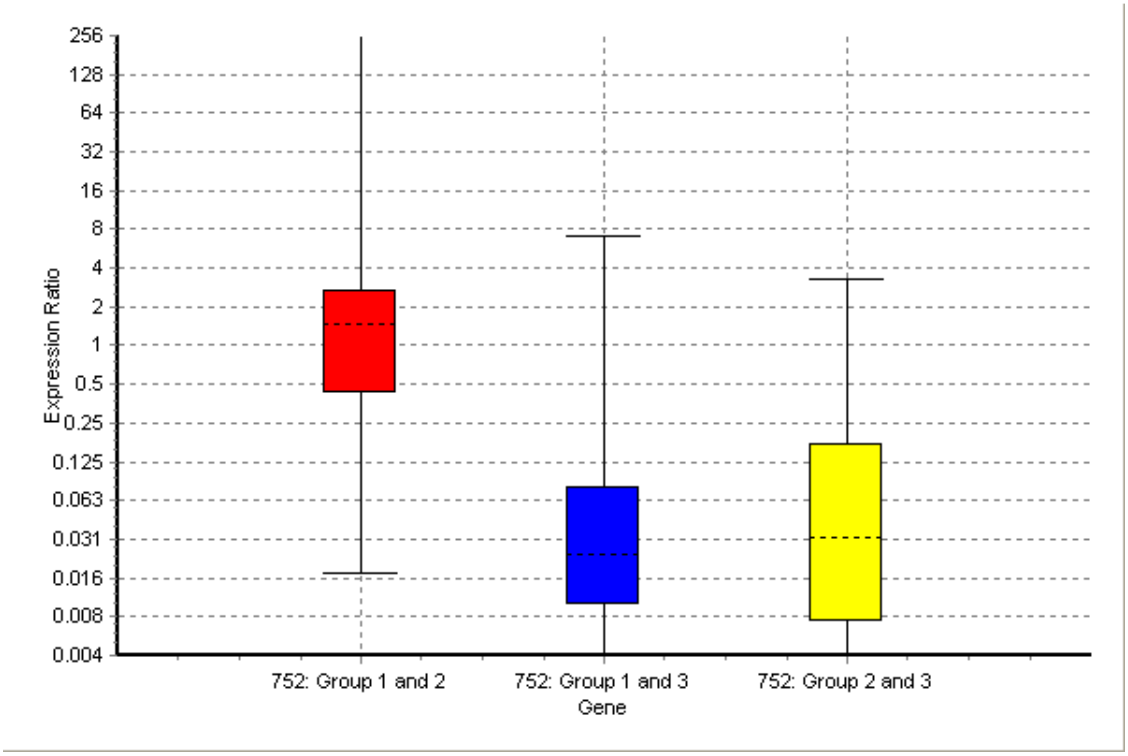

Figure 6.7: The box-and-whisker plot generated in REST 2009 for expression of gene 752, with dashed lines indicating the mean of each box-plot. A significant difference in expression was observed when comparing group 1 and 3; and group 2 and 3.

6.4. Discussion

RNA molecules are quite susceptible to degradation, especially if handling or storage is performed incorrectly [Perez-Novo *et al*. 2005]. Degraded and low quality or contaminated RNA compromises further research. In the current study, the integrity of RNA samples was assessed by absorbance readings and denaturing gel electrophoresis. Absorbance readings indicated all samples to be sufficiently pure and free of contaminants given that absorbance ratio fell into the range of values considered to indicate RNA purity [Fleige and Pffafl 2006; Taylor *et al*. 2010]. One clearly defined band, indicating the 18S rRNA subunit of between 1500bp and 2000bp could be distinguished after denaturing gel electrophoresis of the samples. Molluscs RNA samples that undergo denaturing gel electrophoresis have been reported to display only one band [Serpentini *et al.* 2000] instead of two bands (28S and 18S rRNA) that eukaryotic RNA samples do [Farrell 2005]. Samples therefore appeared to be undegraded and could be used for further applications.

The efficiencies of all genes were well within the acceptable range of 90% to 110%, indicating these genes suitable for downstream quantification. The r and r^2 values, representing how well the regression line (from where the efficiency is calculated) fits the data, was also well within the suggested range of 0.990 and 0.980 [Taylor *et al*. 2010]. Further quantification of target genes with normalization to the reference genes could therefore be undertaken.

Expression differences between the three genes, 238, 135 and 752 for three different *H. midae* age-groups were elucidated by qRT-PCR and normalized with the two reference genes, 126 and 862 by REST 2009. Box-and-whisker plots indicate there to be a high degree of variability in the expression data. However Herrmann and Pfaffl (2005) report that skewed data or the variability of data (as indicated by the box-and-whisker plot) does not invalidate the REST results. The high degree of variability observed in expression data in the current study is most probably due to the limited sample size (five individuals from each age-group) used. Increasing the sample size would most probably eliminate the high degree of variability observed in the expression data.

It was expected that expression of the growth-linked genes would decrease in older age-groups due to decreased growth rates in these animals. However, REST results indicated two of the three genes not to follow the expected pattern of expression in the different age-groups.

Gene 238 was found to be stably expressed within all three groups. This gene is presumed to be a thrombospondin-1 precursor gene. Thrombospondin-1 has been reported to participate in cellular responses to growth factors, cytokines and injury in other species. Trombospondin-1 regulates of cell proliferation, migration and apoptosis resulting in wound healing, inflammation and cell proliferation. These processes involve the formation of multi-protein complexes for which thrombospondin-1 is responsible through specific interactions with growth factors, cytokines, other matrix components and membrane proteins [Lawler 2000; Chen *et al.* 2001]. Thrombospondin-1 most-likely performs a similar function in abalone.

A study by Lucas (2007) indicated a thrombospondin-1 gene to be up-regulated in fast-growing *H. asinina* individuals when compared to diseased slow-growing animals. Also, the initial study by Van der Merwe (2010) indicated this gene to be up-regulated in fast-growing

animals as well. It would therefore be expected that animals in the younger age-groups, exhibiting an accelerated growth rate compared to older animals, would express this gene at a higher frequency than other older groups. The current study however indicates this not to be the case. Both previous studies [Lucas 2007; Van der Merwe 2010] selected larger animals that exhibited obvious larger body mass and growth rate, whereas in the current study animals were grouped and chosen according to age-groups and development. This thrombospondin-1 precursor gene is most-likely not differentially expressed during the different developmental stages of an individual animal, but rather expressed at higher frequency in certain individuals exhibiting an increased growth rate.

Gene 135, the perlustrin gene, is involved in shell formation and biomineralization, and has been reported to bind insulin-like growth factors [Weiss 2001]. Perlustrin was up-regulated in group 3 and 2 in comparison with group 1. Also, group 3 was up-regulated in comparison to group 2. A trend of relative increased expression of this gene can be observed from group 1, the one year old, to group 3, the 4 year olds. A steady increase in relative expression of this gene can be concluded by the expression fold difference between group 1 and 2 (13.029) which is in the same range as that between group 2 and 3 (14.091), while there is a difference of double that between group 1 and 3 (37.692). While it would have been predicted that genes involved in growth would show increased expression during life stages associated with accelerated growth (such as the younger immature animals), this was not indicated by the relative expression detected for this gene. It is known that the ratio of shell to body mass increases as abalone age, most likely due to the linear growth of the shell compared to the exponential growth of the body mass [Reaburn and Edwards 2003]. Although perlustrin has been implicated in shell formation, results from the current study suggest that it could also play a role in soft tissue growth as animals mature. The function and expression of the perlustrin gene has not been fully investigated thus far and further research would be beneficial to elucidate the functioning of this gene.

Gene 752, a gene coding for an insulin related peptide receptor, exhibited the expected decrease in expression from younger immature to older sexually mature animals. Due to the fact that significant differences in expression could be observed between group 1 and 3; and

group 2 and 3, but not between group 1 and 2, it can be assumed that the decreased expression lies with group 3.

Insulin related peptide receptor have also been identified in other mollusc species (*L. stagnalis, Anodanta cygnea, Aplysia californi*a) and have been suggested to consist of a family of closely related receptors [Pertseva *et al*. 1995; Roovers *et al.* 1995; Jonas *et al*. 1996]. Gricourt *et al.* (2006) demonstrated that increased protein synthesis of the gonadal area occurred in response to treatment with insulin-like growth factors in *Crassostrea gigas* (Pacific oyster). Owing to the fact that extraction in this study was carried out on all soft tissue, the specific organ where this gene is expressed cannot be pin-pointed and it was therefore not possible to confirm the possible increased expression of this gene in the gonadal structures. It would however be expected that genes coding for proteins that would be responsible for increased protein synthesis in the gonads would exhibit increased expression in older sexually mature animals. The opposite was found in this study. Gricoult *et al*. (2006) however also reports that insulin-like peptides were found to be highly expressed in maturing oocytes and in developing embryos and larvae. This gene is therefore highly expressed in development of either gonadal structures or immature abalone ova and larvae. The fact that this gene is highly expressed in younger age-groups is therefore not surprising considering the active development and growth of these animals.

The current study therefore has established that genes identified by Van der Merwe (2010) are differentially expressed between different *H. midae* age-groups. Due to fact that the decrease in *H. midae* growth rate is inversely proportional to the expression of the perlustrin gene, it is highly unlikely that a homologous gene construct containing this gene would be effective in increasing the growth rate of transfected abalone.

Although no significant difference in expression for the thrombospondin-1 gene was observed, this gene could still enhance the growth rate of animals containing a homologous construct containing this gene on account of the findings by Van der Merwe (2010) and Lucas (2007). Both these authors found this gene to be highly expressed in individuals with an increased growth rate. Also, this gene was found to be highly expressed in animals of less

than two years old, indicating this gene to be highly expressed in faster growing abalone from an early age.

Although the insulin related peptide receptor gene exhibited the expected decrease in expression with an increase of age of the sample groups (and therefore decrease in growth rate), this does not necessarily signify that that this gene would be useful in transgenic studies. The decrease in the expression of this gene is most likely due to a regulatory process that increases as the animal ages or reaches sexual maturity. The expression of transgenes could therefore also be regulated. If however, this is not the case, this gene could serve as a potential transgene in transfection of *H. midae.* Stable expression of this gene, modulated by insertion of a homologous gene construct, could result in faster growth rates throughout an individual animal's life-span instead of the increased growth rate exhibited in younger animals.

The biochemical pathways and regulation of abalone growth is still poorly understood and therefore assigning the effect one gene would have on the possible cascade responsible for growth is a complicated task. However, the current study has characterized the expression of 3 genes linked to growth that could possible serve as transgenes in future. Future studies will need to concentrate on the elucidation of more abalone genes involved in growth and regulation in an attempt to target these genes for the creation of a homologous gene construct for transfection studies. Organs or tissues exhibiting increased expression of genes related to growth also need to be elucidated in an attempt to grasp the pathway responsible for increases in growth rate. To perform this, it would be necessary to conduct expression studies on individual organs/tissues instead of pooled extractions as was used in this study. Elucidation of the biochemical pathways and regulation thereof would benefit transfection studies greatly.

6.5. References

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7. Conclusion

Several commercially important organisms, including prokaryotes, plants and mammals, have been genetically manipulated [Gama Sosa 2010]. The genetically engineered K1026 strain of *Agrobacterium radiobacter* was the first organism to be released for agricultural disease control in 1988 and has since proven to be highly effective [Jones and Kerr 1989]. Several common crops such as canola, soybean, cotton and tomato have also been genetically modified for characteristics such as herbicide resistance, delayed ripening and virus resistance [Paoletti and Pimentel 1996]. More than 50 plant crops have had the gene *Bt* toxin gene inserted into their genome. This gene is from *Bacillus thuringiensis* and has proven to be an effective toxin to crop pests such as caterpillars and beetles [Skot *et al*. 1990]. However, no transgenic animal has been approved for agricultural production. Transgenic livestock however offer great benefit to the consumer and commercial production industry as long as GMO confinement regulations are adhered to. The current study represents the first report of transfection studies carried out on the South African shellfish species *Haliotis midae* with the eventual aim of creation of transgenic abalone lines. Due to the novel nature of this endeavour, several complementary investigations also needed to be performed and are reported on.

Due to the fact that Wang *et al*. (2004) reported the successful use of a fluorescent protein (EGFP) reporter in *H*. *discus hannai* (and did not report any autofluorescence), it was assumed that autofluorescence would not be encountered in *H. midae.* However, a remarkable degree of autofluorescence was observed in *H. midae* ova, embryos, larvae and spat samples. Not only was fluorescence observed in the emission spectra of the popular fluorescent reporter gene, EGFP, but also in the emission spectra of yellow, cyan and red fluorescent proteins. Larvae were observed to express green fluorescence and proceeded to express localized areas of yellow and red fluorescence as the larvae developed from 2 to 5 days post-fertilization (under FITC filter). Spat also exhibited this localized pattern of fluorescence, although the area of red fluorescence was smaller in comparison with the overall size of the spat in relation with the ratio in the larvae. Red fluorescence in the larvae was concentrated near the dorsal ganglia. It can be assumed that different tissue types would emit a varied fluorescent spectra and that the red and yellow fluorescence emanate from different tissue types in larvae and spat, the area of red fluorescence most probably being the

dorsal ganglia tissues. Future studies would be able to monitor the development of abalone larvae fluorescently by the fluorescent emission and localization. Developmental related research would be able to track fluorescent tissues in abalone without the use of fluorescent reporters. However, fluorescent protein's use as visual reporter genes in *H. midae* is doubtful and therefore this study had to make use of PCR-based validation in larvae.

The fertilization parameters of farmed *H. midae* has not been reported before, nor has the fertilization parameters necessary for small-scale fertilization experiments been reported; this study represents the first published report of the sperm concentration, sperm to ova ratio and period of viability of gametes for small-scale fertilization of *H. midae* (also see Roux 2010 for a complete report on *H. midae* reproduction). Larvae could however only be maintained under laboratory conditions for relatively short periods of time. Due to this fact germ-line transfection and validation thereof was problematic. Future studies aiming at the creation of transgenic abalone beyond the larval stage would benefit greatly from a GMO approved grow-out facility.

Although *H. midae* haemocytes could be successfully maintained for at least a week in culture, the lack of actively proliferation resulted in no expression of transgenes. Abalone research would benefit greatly from the creation of a protocol for the culture of a proliferative cell line. Future research should focus on the addition of specific growth factors and tissue extracts that will be beneficial for specific tissue types. The procedure adopted to harvest, seed and contain each type of tissue should also be adapted to the needs of that tissue.

Results from the current study indicated all the transfection reagents and gene constructs to be relatively non-cytotoxic. An appreciable decrease in larval survival was only observed if transfection reagent treatment was combined with another treatment (such as centrifugation) that exerted environmental stress on the target tissue. The selection antibiotics both exerted a cytotoxic effect within their recommended concentration range. However, the cytoxic effect of neomycin on haemocytes could not be determined due to unsound quantification results most probably caused by a decrease in pH as a result of the antibiotic treatment.

Although the current study confirmed the presence of internalized transgenes in larvae, neither the incorporation into the genome or expression of the transgene could be confirmed.

Future studies that are able to house transfected larvae for longer periods of time, preferable until settling, would be able to carry out further analysis. Expression analysis could be carried out by Northern blotting or qRT-PCR, while characterization of the protein product would be possible by Western blotting, ELISA and immunohistochemistry techniques [Gama Sousa 2010].

Although the mechanism and contributing factors for increased growth in abalone are poorly characterized, characterization of putative growth genes through next generation sequencing and qRT-PCR analysis holds great promise for elucidation of growth genes in abalone [Van der Merwe 2010]. By characterization of three genes linked to an increase in growth, the current study could conclude that two of these genes are differentially expressed between age-groups. However, due to the fact that the pathways responsible for growth in abalone is unknown it could not be concluded what effect expression of these genes in a transfection gene construct would have on overall growth. Future studies would need to undertake transfection of several putative growth genes followed by growth trial to determine whether the transgenes influence abalone growth rate.

Considerable time, effort and funds are necessary for the creation of a GMO. The process of creating a GMO animal destined for commercial use includes optimization of the gene transfer technique, analysis of expression and protein structure, possibly establishing germ-line transfer, phenotypic trials, investigation into human consumption safety and application of approval. Currently consumer scepticism of GMO products deters producers to invest in GMO products and research, even though GMO products hold great advantages for both producer and consumer. Currently, reports indicate that consumers in the Asian markets, the main consumers of abalone products, are sceptical towards acceptance of GMO products. Before GMO products are therefore accepted and established in the commercial environment, consumers will need to adopt a positive attitude towards GMO products [Knight and Gao 2009].

GMO's raises concerns about ecological risk, food safety and bioethics [Nam *et al*. 2001]. Genetic modification of commercial animals has the potential to alter the food products derived from these animals. Stringent evaluation of transgenic food product needs to be

undertaken to ensure that no unexpected and harmful products are produced [Fletcher *et al*. 2004]. The addition of transgenes, specifically a growth gene, has an effect on bodily proportions as well as carcass composition as has been seen in transfected fish species and can alter normal feeding and life functions of transgenics [Nam *et al*. 2001; Lu *et al*. 2002; Wong and Van Eenennaam 2008]. Integration of foreign DNA into the genome of a foreign or homologous gene could have a myriad of effects from having the intended outcome to causing malformation and death of the transgenic organism [Devlin *et al*. 2006].

The environment risk transgenic organisms pose have also caused wide-spread scepticism of transgenesis. The main environmental concerns caused by the possible escape of transgenics is the potential interbreeding of transgenics with wild species and increased competition for ecosystem resources between wild species and escaped GMOs. Adequate containment of GMOs will avert possible ecological disasters. The first line of defence in preventing the escape of GMOs would be to ensure that physical barriers, such as cages, pens and nets are in place. Secondary measures that combat escape in during natural disasters, such as flooding, should also be taken. Reproductive containment is a secondary, but indispensable part of ensuring that GMOs do not cause harm to the environment. Reproductive containment can be achieved by a number of methods that include triploid sterility, knockout of genes that code essential hormones for fertility, RNA interference and prevention of expression of transgenes in the gonads and gametes [see Wong and Van Eenennaam 2008 for an in depth review of these techniques]. The aforementioned techniques needs to undergo stringent testing and further research to ensure their efficacy for their vital role of ensuring reproductive isolation of GMOs and therefore peace of mind.

Several fish species have undergone successful transgenesis as part of aquaculture, industrial or pharmaceutical trials. These species include striped bass, red sea bream, common carp, grass carp, channel carp, Arctic charr, goldfish, mud loach, medaka, pike, Atlantic salmon, tilapia, rainbow trout and zebra fish [Rasmussen and Morrissey 2007]. Integration of this technology for commercial purposes has however been slow. The capacity of fish and other marine organisms to escape and cause environmental disturbances have led to the delay in commercialization of transgenic species [Wong and Van Eenennaam 2008]. Considering that millions of farmed salmon escape every year, the introduction of transgenic

animals into the farming environment could easily result in escaped transgenics. Therefore it is essential to investigate the effect escaped transgenics could have on the ecosystem [McGinnity 2003]. Transgenic animals, especially those that contain a transgenic GH, could have a competitive advantage over non-transgenic conspecifics. Transgenics could also have a reduced susceptibility to predation and an increased reproductive ability [Devlin *et al*. 2006].

Gene transfer to commercially available abalone will however only be possible after several years of research, if ever [Devlin *et al.* 2006]. There is therefore the possibility of consumer acceptance of GMO products at that time [Knight and Gao 2009]. The current study has established a foundation for transfection of *H. midae* and would benefit research into transgenesis of *H. midae* in future. Also, increased production of commercially farmed *H. midae* coupled with a decrease in product prices mediated by transgenesis technology will combat overexploitation of abalone in South Africa.

7.2 References

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8. Appendix

Alsever solution

NaCl	5.6g
Glucose	5.2g
Sodium citrate	2g
PenStrep	2.5ml

Dissolve salts in 250ml milliQ H_2O, then add PenStrep. Adjust pH to 7.5. Filter through 0.2µm into sterilized bottle.

Antibiotic wash solution

Filtered Seawater	483.5ml
PenStrep	10ml
Gentamycin	2.5ml
Amphotericine B	4ml

Adjust pH to 7.4. Filter through 0.2µm into sterilized bottle.

Culture medium

NaCl	10.1g
KCL	0.27g
$CaCl_2$	0.3g
$MgSO_4.7H_2O$	0.5g
$MgCl_2.6H_2O$	1.95g

Dissolve in 500 ml Leibovitz-L15 medium.

Add

PenStrep	5ml
Gentamycin	2.5ml
Amphotericin B	2ml

Adjust pH to between 7.2 and 7.4. Filter
through 0.2µm into sterilized bottle.
Add 10ml Glutamax (or L-Glutamine).

Extraction buffer

2% SDS
0.5M NaCl
2mM EDTA (pH 8.0)
10mM Tris (pH 8.0)
Fill to volume with autoclaved distilled water
Prepare fresh for each use. If SDS precipitates, heat solution to 65℃ before use.

LB medium (broth)

12g/L Tryptone
12g/L Sodium Chloride
6g/L Yeast extract

LB medium (agar)

10g/LTryptone
10g/L Sodium Chloride
5g/L Yeast extract
20g/L Agar

1X MOPS

4.18g 4-Morpholinopropanesulfonic acid (MOPS)
0.68g Sodium acetate
Add 800ml of milliQ water to dissolve salts, before adding 2ml 0.5 M EDTA solution and adjusting pH to 7.0 with 10 M NaOH. Fill to 1L with milliQ water.

TE buffer

10mM Tris (pH 7.5)
1mM EDTA

5X TBE (1L)

54g Tris-Base

27.5g Boric acid

20ml 0.5M EDTA (pH 8.0)

2% Agarose Gel (200ml)

4g Agarose

7µl Ethidium Bromide (10ng/ml)

Fill to volume with 1X TBE before dissolving agarose by microwaving. Add Ethidium Bromide just before gel is poured into geltray.