The effects of cryopreservation on the Pacific oyster *Crassostrea gigas* larval development

Shalini Suneja

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ATTESTATION OF AUTHORSHIP

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

Signed: Shalini Suneja. Date: 5/11/2014

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Dedicated to my dear husband Naresh and my kids

Avi and Rhea.

ABSTRACT

Aquaculture in New Zealand is an important growing export industry and around 66 % of its total production is exported, which is worth NZ $ 400 million every year. Based on the growth strategy of this industry, the aim is to achieve an annual production of U.S $ 1 billion by 2025. In New Zealand, the Pacific oyster *Crassostrea gigas*, is a leading cultivated species with significant exports, along with King salmon and the New Zealand Greenshell™ mussel. Pacific oysters are native to Japan, but now are well established in many parts of the world including New Zealand. Most of the culturing for Pacific oyster in New Zealand is conducted using wild seed, the majority of which are gathered in the Kaipara Harbour on the north-western coast of the North Island. However, this source is highly unreliable, for example recent outbreaks of Ostreid herpesvirus-1 (OsHV-1) led to mass mortality of Pacific oyster larvae in 2010 leaving huge losses within this industry. To overcome these natural disasters and seasonal constraints, use of cryopreserved larvae has been suggested by many scientists. With this method of storing, larvae can be thawed to meet hatchery demands at any time. However, the degree of success using cryopreservation technique is highly variable due to lack of optimum protocols which are highly species specific. Furthermore, little is known about the freezing and cooling effects on the viability of larvae and their subsequent development. Previous studies have been focused on high survival rates just after post-thawing but recently the need to study the effect of cryopreservation on the larval quality over a longer time frame has been recognized.

Before cryopreservation techniques can be applied to oyster larvae, a good understanding of the basic larval development process is necessary under normal conditions. There is lack of literature available on the detailed larval development under

normal hatchery conditions for Pacific oysters. Therefore, the present study is the first to comprehensively describe the various stages of development from D-stage through to settlement of Pacific oyster *Crassostrea gigas* larvae under hatchery practices. To supplement this project, details of the effect of 2 cryoprotectant solutions (CPA) on the larval development from D-stage through to settlement is provided. To achieve the above aims, we used a multi-technique approach involving light microscopy, scanning electron microscopy, immunochemistry and direct visual observations. The findings indicate that these complementary techniques provide the best approach to investigate the larval stages of Pacific oysters. Both cryopreserved and normal (controls) larvae were assessed for survivability, feeding consumption, shell length, shell morphology, organogenesis and neurogenesis at regular intervals.

The methodology of this study included a larval rearing process conducted at Cawthron Aquaculture Park, Nelson. Larval samples were isolated and fixed every alternate day and later transported to Auckland University of Technology on dry ice for further analysis. Raw data for parametric analyses was also supplied by Cawthron Aquaculture Park.

Normal larvae (controls) show a linear downfall in survivability with lowest percentage survivability after 19 dpf, when the pediveligers were approaching metamorphosis. Feeding consumption also varied over the total larval period under investigation and was considerably low after 20 dpf. Shell length show a linear increase before showing some constancy near metamorphosis. Shell morphological observations revealed the presence of a prodissoconch I shell at D-stage with flat hinge and a pitted punctuate region further developing prodissoconch II shell characterised by comarginal growth lines at 5 days post fertilisation (dpf). After progressing to umbo (7-15 days old) stage,

larvae exhibited well developed umbo and a special feature called postero dorsal notch which is characteristic of the members of Family Ostreidae. Later the larvae developed into pediveligers at day 17-19 and finally secrete dissoconch layer during transition from larval period to spat. D-stage larvae exhibited limited organogenesis with development of alimentary canal. With progression of larval days, a protruding velum with well beating cilia and velum retractor muscles were present. Further development led to appearance to posterior and anterior adductor muscles. An important mantle rejection technique called pseudofeces was also observed. In pediveligers development of functional foot and eye spot occurred along with gill rudiment. During settlement, velum was retracted inside the shell indicating metamorphosis. The larval density during settlement period was quiet low because of accidental exposure to higher dose of UV than normal and secondly it indicates healthy competitive settlement behaviour.

During cryopreservation study, larvae were exposed to 2 treatments 10% ethylene glycol + 1% polyvinylpyrrolidone (PVP- 40) + either 0.2 M or 0.4 M trehalose. Three different cooling rates of 0.5, 1, 2˚C min^{-1} between -10 and -35ºC post-holding were investigated. Results show that there were significant differences in survivability, shell length feeding consumption and organogenesis between controls and both the treatments. Comparison between the treatments (0.2 M and 0.4 M trehalose), revealed that larvae exposed to 0.4 M especially with the cooling rate of 1˚C min^{-1} performed exceptionally well. Whereas larvae exposed to 0.2 M trehalose exhibit severe abnormalities with 100% mortality by day 15. Feeding consumption was significantly lower than controls and shell size was considerably small. Shell was more or less oblong giving them oval appearance at early umbo stage rather than circular shape. Organogenesis was the worst effective with severe damage to digestive diverticulum and velum. Moreover, larvae at 5-7 days post fertilisation exhibit D-stage instead of

progressing towards umbo stage. All the internal deformities were indicative of larval death near future. 0.4 M cryopreservation treatment show somewhat satisfactory results at cooling rate of 1˚C min^{-1}, the survivability and feeding consumption were significantly lower than controls but the survived larvae (658 ± 570) show almost similar development but delayed organogenesis and smaller shell size. This delay of organogenesis and other developmental characteristics were indicative of cryoinjuries sustained at the cellular level. However, the degree of cryoinjury was worse in 0.2 M exposed larvae. These results indicates future potential to cryopreserve *Crassostrea gigas* larvae using 0.4 M trehalose as cryopreservative agent provided optimum cooling rate which can enhance larval survivability.

Finally, documentation of larval development in normal as well as in cryopreserved larvae of *Crassostrea gigas* in this project increased our understanding of biology of Pacific oyster larvae and fills the existing gap bridging the cryopreservation studies on relevant species. This study is an important step to reduce the commercial hatchery cultivation cost for this species as it will be easier to distinguish healthy larvae from the abnormal ones.

CHAPTER 1 General

Introduction

1.1 Introduction and literature review

1.1.1 Oysters in general

Oysters belong to the Class Bivalvia. This class is one of the six classes within Phylum Mollusca. True oysters are members of the Family Ostreidae. This family includes the edible oysters, which mainly belongs to Genera *Ostrea*, *Crassostrea* and *Saccostrea*. Morphologically oysters consists of a set of two dissimilar valves of shell hinged together at the anterior end by a ligament (Gosling, 2003). This anterior end is referred to as the umbo. Shell is the primary defence organ as it closes even by minor disturbance in water currents and defend the animal from its predators as well. Organs responsible for its secretion are the shell gland and mantle (Gosling, 2003). Oysters are benthic substrate dwellers attached permanently to the substratum by means of a cement like substance where as in mussels, they are attached with byssus threads (Gosling, 2003). Oysters are filter feeders, resulting in pronounce effects on the quality of water column, which they inhabit, thus referred to as the ecosystem engineers (Padilla, 2010). Individual oysters are capable of filtering up to 50 gallons of water per day and thus presence of oyster reefs can significantly improve the water quality by reducing turbidity and eutrophication (Newell, 2004). Past studies show that oysters and mussels can change the nitrogen levels in estuaries in a significant way, rejuvenating the habitats, (Grabowski & Peterson, 2007). Oysters are suspension or filter feeders using a ciliary action, creating water current that passes between the valves. The water is then drawn between the plicae of their ctenidia which allows both respiration and capturing

of food particles. Food particles are then directed to the labial palps (Ward et al., 1998). However, in mussels, ctenidia plays a limited role in particle selection and the food is simply transported to the labial palps for further processing (Ward et al., 1998).

Oysters are usually euryhaline, which means that they can tolerate a wide range of salinities (10 – 42 %) and hence, explains their cosmopolitan occurrence (Angell, 1986). Maturity is usually attained in first year of life. Being protandric, during first spawning season they act as males by releasing sperm in the water column. As they grow over the next two or three years and develop greater energy reserves, they spawn as females by releasing eggs. However, females can revert back to being males, if there is scarcity of food or if the area is overcrowded. Change of sex is reported at least once from male to female in their lives (Katkansky & Sparks, 1966). Temperature plays a major role in the development of the fertilized oyster egg. Even a minor decrease of 2˚C doubles the time required for the formation of trochophore larvae (Galtsoff, 1964). Reported time for fertilized eggs to reach the veliger stage is 72 hours at 14˚C, and 28 hours at 22˚C (Loosanoff & Davis, 1963). The resulting larvae is pelagic and planktotrophic, feeding on phytoplanktons and grows over a period of 2-3 weeks before undergoing metamorphosis (Kennedy & Breisch, 1981)). Water temperature of 20˚C or greater results in optimal larval development (Magoon & Vining, 1980). Although larvae have tendency to survive at slightly lower water temperatures but this change leads to slow development hence increases their exposure to pelagic predators before attaining sexual maturity (Kennedy & Breisch, 1981).

When oyster larvae grows to shell length of about 0.30mm, they tend to undergo metamorphosis hence losing their free swimming existence and get attached to the bottom, or a hard substrate as spat (Quayle, 1969a). An eye spot develops when the

larval shell length is approximately 250-175 µm, along with the eye spot develops a foot containing byssal gland. Both the foot and the eye serves as defence organs and also aid in locomotion (Loosanoff, 1965). As the time arrives for the larva to settle, the foot gets attached to any suitable solid surface with which it comes into contact preferably other oyster shells. When the contact is made, the larva crawls onto the surface and, attaches by its left valve (Kennedy & Breisch, 1981). At this point, oysters loses its free swimming identity and are called spat. Other vital factors, on which settlement depends includes the presence of oyster shells or other suitable substrata (Crisp, 1967), significant light (Ritchie & Menzel, 1969), and surface irregularities on the substrate (Galtsoff, 1964). It has been reported that oyster larvae prefer to settle and metamorphose on existing oyster shells leading to formation of oyster reefs (Nestlerode et al., 2007). Adults are believed to release conspecific chemical cues which may signal or assist in larval settlement and metamorphosis (Rodriguez et al., 1993).

Of all the oysters, *Crassostrea sp* are the most important commercial species and have the greatest development potential due to their wide tolerance of estuarine conditions of temperature fluctuations and salinity, exhibiting high fecundity resulting in abundant spatfall (Angell, 1986). Keeping in mind the aquaculture point of view, *Crassostrea gigas* is one of the most important species. This species is available world-wide and is very well established in New Zealand (Dinamani, 1987).

Crassostrea gigas (Thunberg) is an oyster species native to Japan, but due to its wide environmental tolerance and high growth rate, it has established itself in most parts of the world. In New Zealand, the Pacific oyster was unintentially introduced in late 1960's (Dinamani, 1971), most likely through the hulls of ships, but now it has outcompeted the Sydney rock oyster (*Saccostrea glomerata*), which occurs naturally in

intertidal areas in North Island (Dinamani, 1987). There are 39 species belonging to genus *Crassostrea*, but *Crassostrea gigas* is the only species from this genus found in New Zealand.

1.1.2 About Crassostrea gigas

Crassostrea gigas was named by a Swedish naturalist, Carl Peter Thunberg in 1795. Pacific oyster (*Crassostrea gigas*), also known as Giant oyster, Giant Pacific oyster,, Portuguese oyster, Immigrant oyster, Japanese oyster, etc., is a bivalve mollusc, belongs to the family Ostreidae. They are soft-bodied animals enclosed in shell. The shell comprises of two unequal valves, extremely rough and laminated hinged together by the ligament. The lower valve is deeply cupped and the upper valve is slightly convexed (Harding & Mann, 2006; Nehring, 2006), with the varying shape, depending on the environment and the nature of substratum (Nehring, 2006; Pauley et al., 1988). Oysters cementing on soft substrate have relatively less laminas giving more or less smooth appearance to the shell than those who settle on hard substrate. Shell colour is usually whitish bearing purple streaks and spots which radiates away from the umbo. The internal organs in the shell have white appearance, with a dark single muscle scar (Pauley et al., 1988). Mature specimens can vary from 80 – 400 mm in length. Pacific oyster can tolerate a wide range of temperature from -1.8˚ to 35˚C. It can also tolerate wide range of salinity as well (Nehring, 2006), hence can be found in intertidal and sub-tidal zones.

Figure 1.1.*Crassostrea gigas* showing external shell morphology, Source MPI.

Nomenclature and Taxonomy:

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Bivalvia
Order:	Ostreoida
Family:	Ostreidae
Genus:	*Crassostrea*
Species:	*gigas*
Common name:	Pacific oyster
Other names:	Giant oyster, Japanese oyster.

Pacific oysters are protandrous hermaphrodites, maturing first as males and releasing sperm in water column, but as they grow for the next two or three years they conserve enough energy to release eggs acting as females (Fretter & Graham, 1964; Guo et al., 1998). Females, however can also revert back to males if there is scarcity of food or if the place is overcrowded. But change of sex from male to female surely occurs at least once at some point of life (Katkansky & Sparks, 1966). Synchronous spawning generally occurs at around 20˚C. The species is very fecund with each mature female releasing 50- 200 million eggs in a single spawning. A study by Dinamani (1987) indicated that in New Zealand *Crassostrea gigas* can reach sexual maturity in as young as 6 months, compared to 2-3 years in British Columbia (Quayle, 1969a). Spawning events occur in New Zealand from early spring until late summer mainly between December and March (Dinamani, 1987). *Crassostrea gigas* are quiet flexible in the reproductive process as synchronous spawning can occur at as low as 15˚C (Loosanoff & Davis, 1950), rather than 18˚C, which is considered as best optimal temperature for the release of gametes (Mann, 1979). When oyster larvae attains an approximate shell length of 0.30mm, their free swimming existence ends and they get attach to the bottom, or a hard substrate as spat (Quayle, 1969a). This metamorphosis is accompanied by some marked morphological changes, such as the degeneration of the velum and foot as well as the anterior adductor muscle, and development of the enlarged set of gills (Quayle, 1969a).

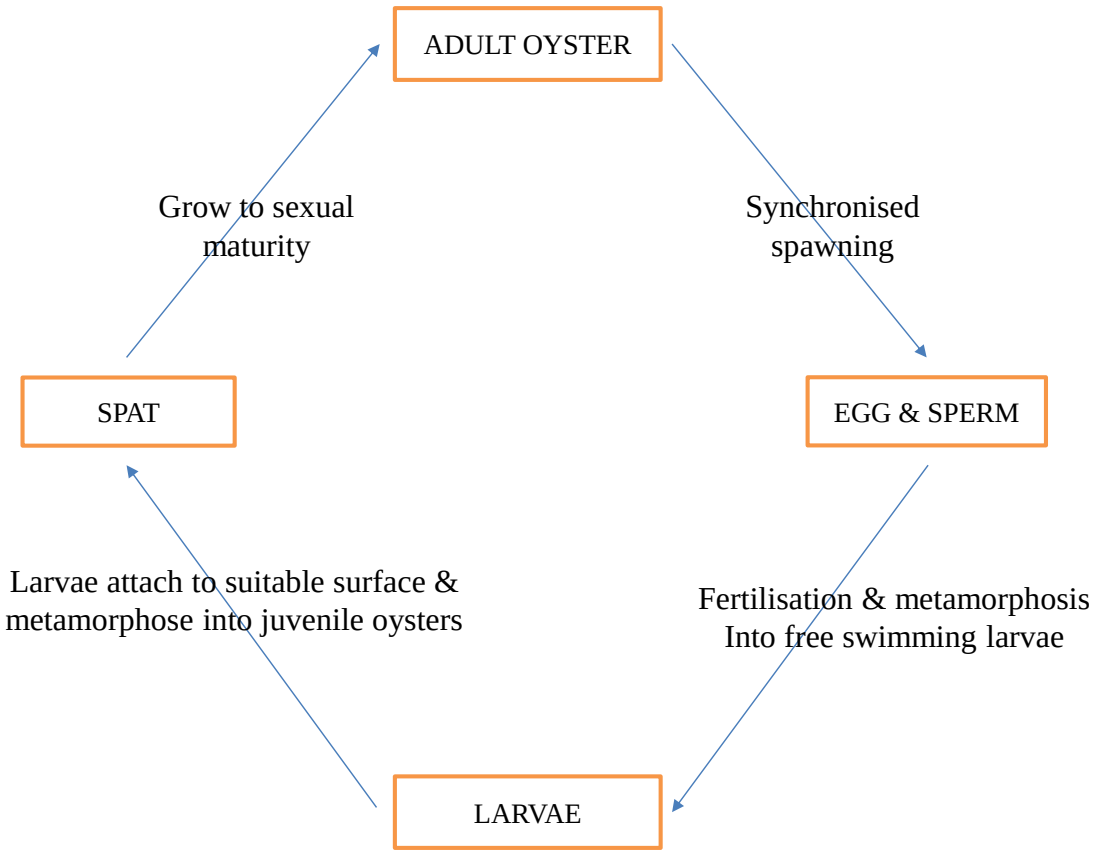

Figure 1.2.Simplified life-cycle of the Pacific oyster (*Crassostrea gigas*).modified from Bell (2005)

1.1.3 Global and national aquaculture importance

The New Zealand aquaculture industry began in the mid-1960s with marine farming of oysters and then mussels, typically by small private farms. It quickly established a domestic market and started its operations into export markets in the 1970s. During the 1990s, global competition in seafood intensified, resulting in increased efforts to achieve high demand in production. With pressures on the market, the market for oysters and mussels began to diversify, along with salmon. The aquaculture sector in New Zealand has grown steadily over the past 40 years and is estimated to have revenue in excess of $ 400 million, with a target of reaching $ 1 billion by 2025 as a government's goal to increase the seafood sector (Ministry for Primary Industries, 2014).

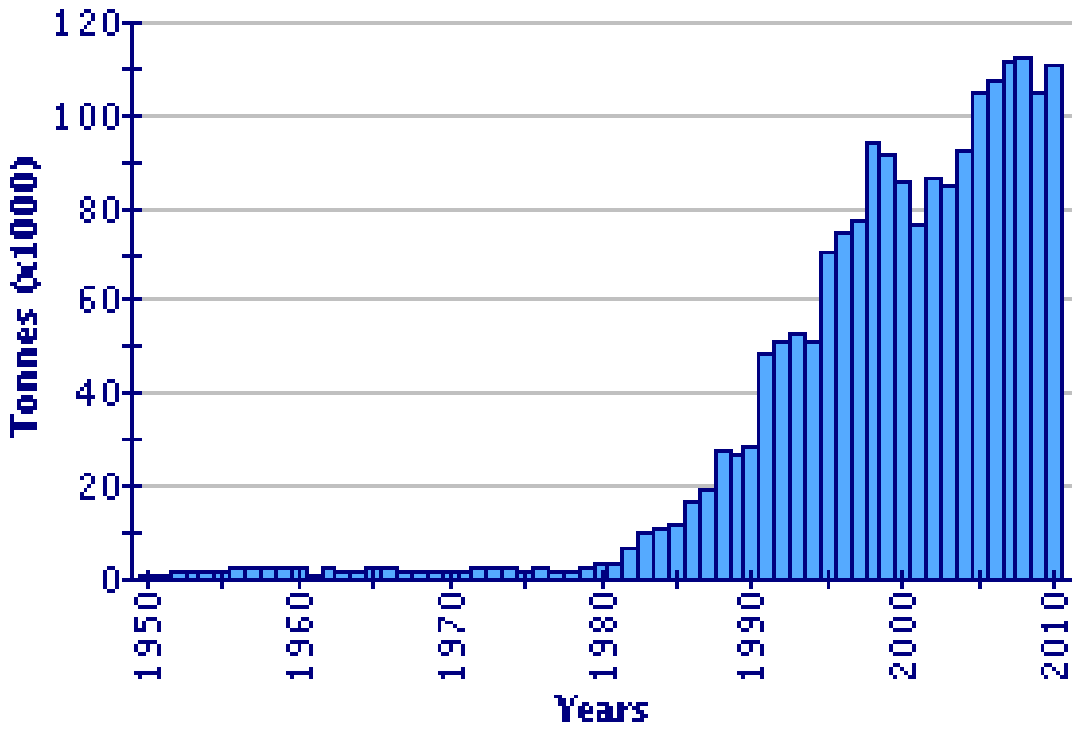

Figure 1.3.Reported aquaculture production in NZ from 1950. Source FAO fishery statistics (2014).

Currently, New Zealand aquaculture sector is a $ 390 million per annum based on three species, NZ Greenshell mussels™, Pacific oysters and King salmon. Of these three species, Pacific oyster alone is responsible for a revenue collection of NZ $ 35,000 per annum per hectare (Ministry for Primary Industries, 2014). High water quality and low endemic biosecurity threats owing to high biosecurity standards have significantly improved the productivity, quality and hence, acceptability for oysters in domestic as well as in international market. There are total 230 oyster farms, covering 750 hectares of marine water. These farms produce around 3,300 tonnes of oysters worth NZ $32 million, which comprised of NZ $14 million domestic market and $.NZ $18 million export market. The Single largest export destination for Pacific oysters from New Zealand in 2005 was Australia (U.S $ 5 million), followed by Japan and U.S.A. These markets are mostly supplied with fresh or frozen, half-shell oysters (FAO, 2014) depending on the demand of international market.

1.1.4 Oyster farming in New Zealand

Pacific oyster farming in New Zealand has reached to a point where it provides direct and indirect employment to thousands of skilled workers and resources are feeling pressurised to maintain the current development with increase in future production. To maintain industry growth, security of juvenile oyster (spat) supply and productivity gains within the existing farm leases, are becoming important goals (Bell, 2005). Oyster farming in New Zealand began in 1960's with the establishment of government trial farms in the Mahurangi harbour and Bay of Islands, producing New Zealand rock oyster and Pacific oyster. The oysters were then cultivated on wooden or fibrolite battens attached to the long rails in the intertidal zone (New Zealand Marine Department & Curtin, 1968).

In New Zealand, oyster farming was established primarily by small scale farmers. At that time the industry struggled because of inconsistency with the natural spat supply, as farmers were dependent on collection of wild spat only. In an attempt to support the industry, the New Zealand Ministry of Agriculture and Fisheries (MAF) now called as Ministry of Primary Industries (MPI), investigated the hatchery practices (Ministry of Agriculture and Fisheries & Curtin, 1979). The attempt of hatchery was successful in producing spat for both the Pacific oyster as well as New Zealand rock oyster, but the technology was far beyond the farmer's limited financial conditions and resulting seeds could not be used in the traditional rack culture system which was preferred by majority of the industry at that time (Ministry of Agriculture and Fisheries & Curtin, 1979). At this point, the Pacific oyster was getting established in New Zealand, and farmers who were already facing problems with slow growing New Zealand rock oyster, finally replaced them. As a result *Crassostrea gigas* became one of the major aquaculture species due to its fast growing nature (Bell, 2005).

By the late 1970's, New Zealand oyster farming had shifted exclusively to the Pacific oysters. Currently, there are 230 oyster farms. Majority of them are located in Northland, Auckland and Coromandel regions at the top of New Zealand's North Island, which accounts for the most of the Pacific oyster production. Some farms exist in the Northern part of the South Island as well (Ministry for Primary Industries, 2014). However, the threat of spatfall failure still exists and the financial implications are now much greater than they were at the beginning. Such an example was the closure of Kaipara Harbour in 2000/2001, due to a toxic algal bloom (*Gymnodinium cetanatum*), which delayed the spat collection (Bell, 2005). Another most recent tragedy was the attack of Osterid herpes (OsHV-1) virus, which completely washed out the country's oyster stock in 2010 (Cawthron, 2013). More than 90 % spat was infected with this

virus resulting in job losses, factory closure and an overall drop in production of 50-60%. Apart from algal bloom and virus infections, there are other natural obstacles in the spat production which includes oil spills, parasites or climate change which could result in the depletion of broodstock populations. Due to this unreliability being felt continuously by the farmers, leading to the necessity of having an alternative supply of oyster spat from large scale commercial hatchery.

1.1.5 Hatchery rearing of Crassostrea gigas

Much of New Zealand's commercial oyster spat comes from Cawthron Institute in Nelson, New Zealand (an independently owned Research Centre), which can selectively breed spat to specifications. Cawthron has established selective breeding programmes for Pacific oysters as well as mussels bringing international recognition. One in three oysters harvested by the New Zealand industry starts its life in Cawthron (Cawthron, 2012). The adult oysters are chosen selectively for their quality, and induced to spawn under favourable conditions. The gametes are collected carefully and fertilised, the resulted embryos develops into swimming veligers under controlled environment. These larvae are fed with mixed diets of microalgae most suitable for growth and are reared until the spat formation and then are transported to different farms according to demand.

Some advantages of hatchery spat over the wild spat, which compels us to develop hatchery rearing conditions are the faster and uniform growth, high quality meat and availability of single seed spat. Oysters grown from single seed spat do not attach to the growing trays, hence harvesting and sorting is much more convenient as compared to wild spat (Cawthron, 2007). Many potential breeding lines have been recognised by Cawthron's selective breeding programme for further development of oyster products for domestic and foreign market

Cawthron is developing methods for the cryopreservation of sperm, eggs and larvae of shellfish using the technique of selective breeding, such those programmes are already in function for the Greenshell mussel™ and the Pacific oyster. Cryopreservation is basically the storage of live material at temperatures so low that all biological processes are suspended (-196˚C). This process enables the storage of sperm and eggs from

superior stock and thawing them in future when required. This practise overcome various constraints such as conditioning the adults for synchronous spawning and seasonal limitations The sperm and the eggs can be stored for hundreds of years way beyond the lifespan of the parent. Cryopreservation also enables hatcheries to produce juveniles (spat) all the year around, reducing the cost of artificial spawning out of season and is widely practised in animal husbandry (Paredes et al., 2012). Researchers at Cawthron have successfully cryopreserved the sperm (Adams et al., 2004) and oocytes (Tervit et al., 2005) of *Crassostrea gigas* so far and the efforts to cryopreserve larvae of this species is progressing well.

Paredes et al. (2013) attempted to test the toxicity of cryoprotective agents on the trochophore larvae of *Crassostrea gigas* and *Perna canaliculus*. The study found that 60.0 ± 6.7% of the 14h old oyster larvae were successfully transformed to normal D-stage larvae. The resulting mortality is suggested to be affiliated with cryoinjuries during the thawing or cooling process. However, no studies have been done so far to investigate the effect of those damages on the subsequent larval development of *Crassostrea gigas*. This study therefore aims to investigate the effects of cryopreservation on subsequent larval development by cryopreserving D-stage larvae, and also to describe the normal larval development of *Crassostrea gigas* under hatchery conditions.

Therefore the aims of this thesis are:

AIM 1: To describe the larval development of *Crassostrea gigas* from day 1 through to post-settlement under normal hatchery conditions.

The objectives are:

- To describe shell morphology of larvae through scanning electron microscopy, and light microscopy
- To describe survivability and feeding patterns of developing larvae over the total rearing period of 22 days.
- To describe organogenesis processes through light microscopy
- To describe neurogenesis through immunochemistry.

AIM 2: To identify the effects of cryopreservation (cryoprotectants and freezing rate) on larval development of *Crassostrea gigas* from day 1 through to post-settlement.

The objectives are:

- To describe shell morphology of cryopreserved larvae using scanning electron microscopy and light microscopy.
- To describe survivability and feeding patterns of cryopreserved larvae over the total rearing period of 22 days.
- To describe organogenesis processes in cryopreserved larvae using light microscopy.
- To describe neurogenesis in cryopreserved larvae using immunochemistry.

CHAPTER 2 Larval Development

2.1 Abstract

A multidisciplinary approach was used in the present study to investigate the ontogenetic and neuronal development of the Pacific oyster, *Crassostrea gigas* larvae. There are only a few studies which have described the morphological and behavioural aspects of Pacific oyster larvae and even fewer studies which have focused on organogenesis and neurogenesis. Therefore, a comprehensive study on larval organogenesis, shell morphology, central and peripheral nervous system along with survival and feeding data were collected for this species. The study involved experiments under normal hatchery conditions, over a 22-day rearing period. The entire larval rearing process was conducted at the Cawthron Aquaculture Park, Nelson. During the rearing process, the larval samples were isolated every alternate day to record various developmental characteristics including, shell length, percent survival, and feeding consumption. Larval samples collected at regular intervals were fixed and stored until they could be further analysed. Larval sample analyses included examination of shell morphology, organogenesis and neurogenesis over the 22 days rearing period. Analysis of shell morphology using SEM revealed that D-stage larvae have a flat hinge with pitted punctuate prodissoconch I (PI) shell. As the larvae grow PI is followed closely by Prodissoconch II (PII) shell with comarginal growth lines and a well-developed velum is protruding out with large number of active cilia. As the larvae reach umbo stage, there is further secretion of PII shell characterise by the presence of postero dorsal notch, which is characteristic feature of Family Ostreidae. At the pediveliger stage, the larvae start preparing for metamorphosis. The velum is retracted

inside and dissoconch layer is secreted by the mantle, which helps the larvae to attach to the substratum.

Light microscopy revealed complex changes that occurred inside the shell during larval rearing. D-stage larvae exhibited limited organogenesis with alimentary system starting to develop. At the early umbo stage, larvae assumed more or less a circular shape with a developing umbo and velum. Further organogenesis occurred as the larvae reached the umbo stage, the velum appeared with large number of beating cilia. A well-developed anterior and posterior adductor muscle, velum retractor muscles and alimentary canal were also present. At the pediveliger stage, the gill rudiment started to appear along with an eye spot and functional foot. After this, larvae underwent rapid morphogenesis. The velum retracted inside and the shell closed. The functional foot and eye help the larvae to find suitable substratum. After attachment, the foot degenerated and the larvae attached to the substratum permanently with a cement like substance. Neurogenic study revealed the development of nervous system, which started at the D-stage, but due to less absorption of FMRFamide by the larval cells, neurogenesis could not be described in detail in the present study. However, in the pediveliger larvae, the foot exhibited high immunoradioactivity as it is highly innervated by neurons.

The raw data collected to record the population dynamic parameters, during the 22-day rearing period was also analysed further. The percent survival linearly decreased over time, with lower survivability after 19 dpf, maybe due to the fact that the larvae were accidentally exposed to a high dose of UV than normal. Feeding consumption also showed variations at different intervals of time and was considerably lower after 20 dpf, before showing peak consumptions at day 5, 11 and 17 (prior to metamorphosis). Shell length show a positive linear trend up until day 17, after which it become somewhat

constant, but this deviation is quiet obvious as the larvae nears metamorphosis. A few competent larvae then progressed onto the post-settlement stage and attached to the substratum, indicating the completion of the larval period and successful metamorphosis.

2.2 Introduction and literature review

2.2.1 Bivalves in general

The Class Bivalvia is the second largest class in the Phylum Mollusca, and includes great variety of clams, mussels, oysters and scallops, both fresh water as well as marine (Ponder & Lindberg, 2008). There are approximately 200,000 living species in this phylum (Ponder & Lindberg, 2008) of which more than 8000 species were believed to belong to the Class Bivalvia (Boss, 1982), but now more than 15,000 species have been recognised (National Research Council, 2004). Bivalves are generally known for soft bodied animals enclosed in mantle cavity protected by shell. The bivalve mantle is reported to perform variety of important functions such as shell formation, storage of energy reserves and receptor of external stimuli such as predators and presence of unsuitable conditions (Beninger & Le Pennec, 1991). Bivalves play vital roles in the functioning of marine, freshwater as well as in terrestrial ecosystems. Their role is not only confined to ecosystems, but they have major effects on humans, primarily as food sources and medicinal use and ornamental use. They also act as vectors of parasites and bio-fouling agents. Many bivalves act as ideal laboratory material to study neurology, reproductive physiology, ocean acidification and climate change. Therefore, there is vast literature focussed on bivalve larval development. Huxley (1883) provided first

description of the bivalve development. Since then various bivalves which are of economic and commercial importance have been examined to assist with aquaculture production, such as oysters (Dinamani, 1971; Ellis & Kempf, 2011; Elston et al., 1999; Gwo, 1995) and mussels (Alfaro, 2005; Buchanan & Babcock, 1997). Bivalves assist in maintaining balanced ecosystems in various ways. They act as nature's water filters because of their filter feeding habit and they also aid in regulating phytoplankton levels by feeding on them, and helps in diversifying the benthic population providing shelter to them. Though variety of bivalves are of commercial and economic importance but oysters in specific have seek a lot of attention as their presence indicates the water quality and type of fauna present in entire estuarine habitat (Ellis & Kempf, 2011). Moreover, bivalves especially shellfish holds a special place in seafood industry as it's a great source of revenue. Therefore, the research work on bivalves and their larvae attracts a lot of attention especially the commercial viable species.

2.2.2 Bivalve larval development

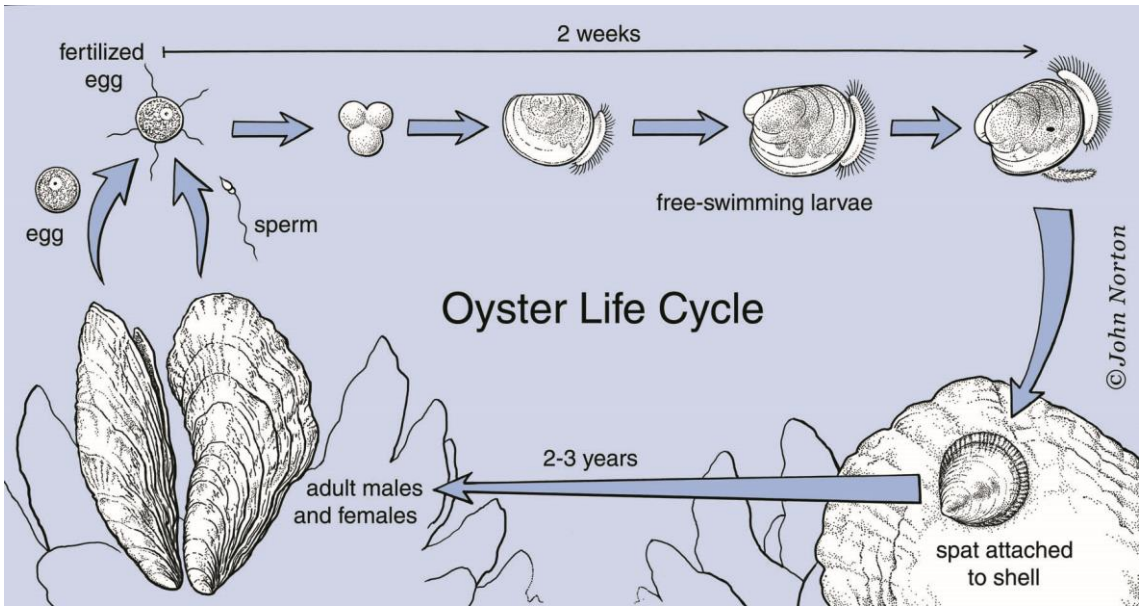

Figure 2.1.General oyster life cycle as illustrated by John Norton (Science-Art.com).

In general, bivalve life cycle begins with fertilization, which may result from cross fertilisation or the self-fertilisation, internal or external, depending on the species, followed by an embryonic period (Fig. 2.1). Cross fertilisation is the common method found in the hermaphrodites However, self-fertilization has also been documented (Heller, 1993). Some oyster species practise external fertilisation, such as *Crassostrea virginica* and *Crassostrea gigas*, as they expel their gametes into the water column (Waller, 1981), whereas *Ostrea edulis* practises internal fertilisation producing spermatozoa which enter into the female brood chamber where the eggs are fertilized (Waller, 1981). This process of releasing the gametes in water is termed as spawning, which is quiet synchronised and is favoured by suitable water conditions like temperature, water currents and salinity. Though these animals are highly fecund releasing large number of sperm and eggs but the fate of fertilisation highly depends on abundance of conditioned adults, and external environmental factors like temperature,

water currents and salinity. The resulting egg (planktotrophic/ lecithotrophic) varies in size and is generally the indicator of the mode of development (Jaeckle, 1995). Planktotrophic eggs are generally small (40-85 µm) as they do not have much energy reserves but adequate enough for the larval development. These larvae must begin to feed immediately upon development of feeding structures since they rapidly deplete their relatively insignificant yolk stores, while lecithotrophic eggs are large (90-300 µm) as they are high in energy reservoirs, called egg yolk (Gosling, 2003). These large yolky eggs develop into non-feeding larvae which usually lack feeding structures. After successful fertilisation, embryos are formed which subsequently go through the series a spiral cleavages resulting in formation of micromeres (Gosling, 2003), followed by the blastula, gastrula and trochophore stages. By the time planktotrophic eggs reach trochophore stage, all the energy reserves are used up so the larvae immediately develops a velum feeding apparatus, mouth, stomach and starts feeding (Zardus & Martel, 2002). This trochophore stage is highly motile as it has well developed cilia which helps the larvae to swim along the water current (Ellis & Kempf, 2011). As soon as trochophore stage arrives, two major ciliary bands develop which enables the larvae to swim. The ciliary band present anterior to the blastopore is called prototroch, and the one present near the anus is called telotroch (Nielsen, 2004). In addition to these ciliary bands, an apical tuft is present which is an outgrowth of cells of the apical sense organ (ASO) or apical ganglion (Mouëza et al., 2006). The apical sense organ is also described as a sensory receptor for light, chemicals and orientation, hence helps the larvae to adjust with the external environment during the D-stage (Nielsen, 2004). Recent investigations by Hadfield et al. (2000) have established that the apical ganglion contains the sensory receptor for the settlement cue. The trochophore also has a large invagination on the dorsal side which represents the shell gland. The shell gland is

composed of multiple cell types that produce the periostracum, hinge and finally the calcified prodissoconch, the larval shell (Mouëza et al., 2006).

The trochophore then undergoes further differentiation and develops into a veliger larva. The early veliger stage is also called D-stage larvae that possess a functioning velum which helps in feeding and also aids in swimming. The larvae first begin to feed on suspended phytoplankton and then undergo organogenesis as it develops further. Development of hinge shell soon after attaining D-shape is characteristic of the Subclasses Pteriomorpha and Heterodonta (Zardus & Martel, 2002). Later, the larva becomes laterally compressed. This compression results in formation of velum by displacement of the prototroch (Mouëza et al., 2006), a well grown velum later develops crystalline sac and an adductor muscle, which helps regulate opening and closing of the valves (Zardus & Martel, 2002). As the organogenesis proceeds velum retractor muscles are developed, there are four pairs of velum extending muscles and three pairs of velum retractor muscles which are attached to the posterior body wall (Cragg, 1985). These muscles aids in extending and retracting velum inside the valves. Velum is composed of different whorls, with presence of ciliary bands on its margins. These ciliary bands aids in swimming and helps collecting particles suspended in water column for ingestion (Zardus & Martel, 2002).

After veliger stage, follows the umbo stage. At this stage, a well-developed umbo is formed with presence of larval foot, though the foot is not specialized for crawling on substratum at initial stages. During this time, a pigmented eye spot also develops at the base of velum (Galtsoff, 1964), although in some cases eye spot may not always be clearly detectable until larvae reaches 15 days post fertilisation. Upon further development, pediveliger stage is reached. At this larval stage, major morphological

characteristic is the larval foot as it develops a metapodium and toe or propodium for crawling on benthos (Mouëza et al., 2006; Waller, 1981). Foot maturation allows larva to settle and metamorphose on the substratum. This developmental process is followed closely by reabsorption of velum into larval shell and finally attachment of larvae onto the substrate with help of mucoid filaments or byssal threads or cement like substance depending on the species. This development indicates beginning of benthic life, statocysts develops later in this larval stage and are located on either side of the pedal ganglion, statocysts assist with orientation of foot and mouth in newly settled larvae producing a juvenile or spat, which eventually matures into a reproductive adult (Zardus & Martel, 2002). Rate of bivalve larval growth is highly influenced by genetic factors, nutrition, and culture conditions such as temperature (Doroudi & Southgate, 2003).

Although the above description represents a general bivalve life cycle, but past studies reveal some variations. The most interesting one is reported in freshwater mussels life cycle, where a parasitic larval stage called Glochidium is observed (Haag & Warren, 1997).

2.2.3 Bivalve larval shell morphology

The main purpose behind describing larval shell development is to gather some basic knowledge, which can help in general larval identification for assessing wild populations. Past studies have shown that the shell morphology of various bivalve species has been extensively described from the shell formation during trochophore through to juveniles using Scanning electron microscopy (SEM) and light microscopy (Acarli & Lok, 2009; Dinamani, 1973; Doroudi & Southgate, 2003; Galtsoff, 1964; Rusk, 2012), as shell morphology is the primary characteristic used in species identification in larval bivalves. Closely related bivalve species have a similar larval shell morphology with minor differences as we move on to next species. These minor differences in shell morphology acts as the criteria used to differentiate larval bivalves at the species level. Therefore, any change in shape or inaccuracy of measurements during SEM, even minor ones could result in confusion among species leading to wrong identification (Fuller et al., 1989).

SEM provides more specific visual approach to determine prodissoconch layers, comarginal growth rings, and surface shell patterns that would not normally be visible with a limited scope of compound microscope. During the embryo stage, the shell field gets activated, which in turn initiates the ectodermal cells which represents shell gland to start forming the first larval shell (Kniprath, 1980). The shell gland is composed of multiple cell types that produce the periostracum, hinge and ultimately the calcified prodissoconch that is referred to as the larval shell. The secretion of a shell gland continues for the entire life of the animal, from the very start of larval stage until the death of animal (Mouëza et al., 2006; Weiss et al., 2002). Bivalve species with planktotrophic larval stages, such as oysters, secretes 2 layers of shells in succession,

initially a layer called prodissoconch I (PI) followed by its successor, prodissoconch II (PII) layer (Ockelmann, 1965). The mode of secretion for these two layers is totally different. As the larvae transits from trochophore stage to D-stage, PI comes into existence. This layer is secreted by the shell gland and mantle epithelium (Carriker & Palmer, 1979; Waller, 1981). At this stage, the larvae is compressed laterally, the hinge is flat more or less D-shaped. Hence, the D-shaped larva gets its name from its shape (Christo et al., 2010; Zardus & Martel, 2002). Stellate radial lines extending towards the transition zone of PI and PII along with a centralised pitted region characterise the PI shell region. (Foighil, 1986). As the organogenesis takes place and the larva undergo further development to reach veliger stage, the second larval shell layer PII develops, which is entirely secreted by the mantle edge. This change in mode of secretion from shell gland to mantle is gradual and it occurs well before the occurrence of prodissoconch I/II boundary (Waller, 1981). PII layer is characterised by the presence of comarginal growth lines, which are very closely concentrated, PII region can be easily differentiated from PI region as the texture of both the layers is very much different from each other. The PII layer serves important function of preventing shear between two closed valves, as the margins fits together in tongue-in-groove fashion (Carriker & Palmer, 1979; Ockelmann, 1965; Waller, 1981).

There is another important taxonomic development as the larvae secretes PII, hinge teeth and the ligament. Study of hinge teeth is quiet useful in taxonomic determinations at species level (Christo et al., 2010; Zardus & Martel, 2002). This is because different species have different numbers of teeth and some lack them altogether, but shell morphology for all of them is basically the same. The only difference can be observed by SEM observations of hinge teeth (Christo et al., 2010). Hinge teeth bear curved ridges on their sides that provide shear to partially closed shells as in adults (Waller,

1981). Another important feature which is unique for members of the Family Ostreidae is the development of a postero dorsal notch at this stage (Malchus, 2000; Waller, 1978). This notch positions itself exactly adjacent to post-anal ciliary tuft and it disappears at prodissoconch/dissoconch boundary. Reason for this disappearance is metamorphosis, when the larva undergo metamorphosis, it loses its post-anal tuft (Waller, 1981) Moreover, the prodissoconch of many oysters, especially those belonging to Ostreidae, are more asymmetrical than those of most bivalves and they generally possess a distinctly wavy rather than flat commissural plane (Malchus, 2000).

Size of the Prodissoconch I and II secretion varies among taxa depending on whether the larva undergoes planktotrophic or non-planktotrophic development. In the former case, larva exhibits relatively smaller PI (70-150 µm) compared to PII (200-600 µm). However, in case of non-planktotrophic larva, the larva possess relatively large PI (135-500µm) compared to PII, which can be reduced or may be completely absent (Ockelmann, 1965). The above fact is also supported in family Ostreidae. The size of PI/PII boundary has been shown to correlate with size of the egg and is important indicator of larval development and its capability of dispersal (Ockelmann, 1965).

The secretion of PII layer continues for the entire life of larva, until it has developed enough and undergo metamorphosis and get attached to a suitable substratum preferably another oyster shell (Chanley, 1971). At this stage, the larva is referred as a pediveliger larva with a well-developed umbo and a functional foot, which helps the larva in locomotion to find a suitable substratum (Gosling, 2003). As the larva transits from floating pediveliger stage to the attached benthic stage, the velum gets reabsorbed and mantle secretes another shell layer called the dissoconch (D), consisting of comarginal lines between transition zone of PII and the dissoconch layer (Carriker & Palmer, 1979;

Chanley, 1971). After attachment and successful metamorphosis, larva is finally called as post-larva or spat or juveniles. They spend rest of their life attaching to the substratum and growing their shell size through-out (Gosling, 2003).

2.2.4 Bivalve larval nervous system

Any study directed towards an understanding of bivalve larval nervous system assist in defining larval behaviours and structure and function of various organs which play vital roles in settling and metamorphosis. This aim can be achieved by doing immunological studies on the bivalve larva using confocal microscopy. This advanced technique facilitates identification of physiological changes and neuronal development of the larva (Ellis & Kempf, 2011). Confocal microscopy allows in-depth visualisations of an organism, revealing optical 3D images of complex larval systems. Labelling the larva with neuronal fluorescent labelling such as FMRFamide and catecholamine immunoreactivity and observing them under confocal microscope may reveal structures which cannot be observed with a regular compound microscope. Hence, this technique has been extensively used to study the neuromuscular development and immunochemistry of molluscs (Kristof & Klussmann-Kolb, 2010; Todt et al., 2008). Unfortunately, literature lacks proper analysis of nervous system structure and ontogeny within the bivalves larvae (Ellis & Kempf, 2011).

Considering a general bivalve adult nervous system, it consists of paired cerebro-pleural, pedal and visceral ganglia, which are connected by commissures and cerebro-visceral and cerebro-pedal connectives (Brusca et al., 1990). Various studies have thrown light on adult nervous system of molluscs, with gastropods receiving special attention, but only a few studies have focused on the nervous system of bivalve larvae. A study by Erdmann (1935) on larval nervous system of the oyster *Ostrea edulis* suggested that pediveliger nervous system is highly complex (Fig. 2.2). His findings suggested that there is a presence of an apical sensory organ (ASO) in pre-metamorphic *Ostrea edulis.* This ASO is positioned between two velar lobes with two cerebral

ganglia positioned posterior to it. From each cerebral ganglion leads short connectives which extends to respective pleural ganglia. The larvae also possessed paired pedal and visceral ganglia with pleural-visceral connectives on the left and right sides. All the paired ganglia were joined to each other with commissures.

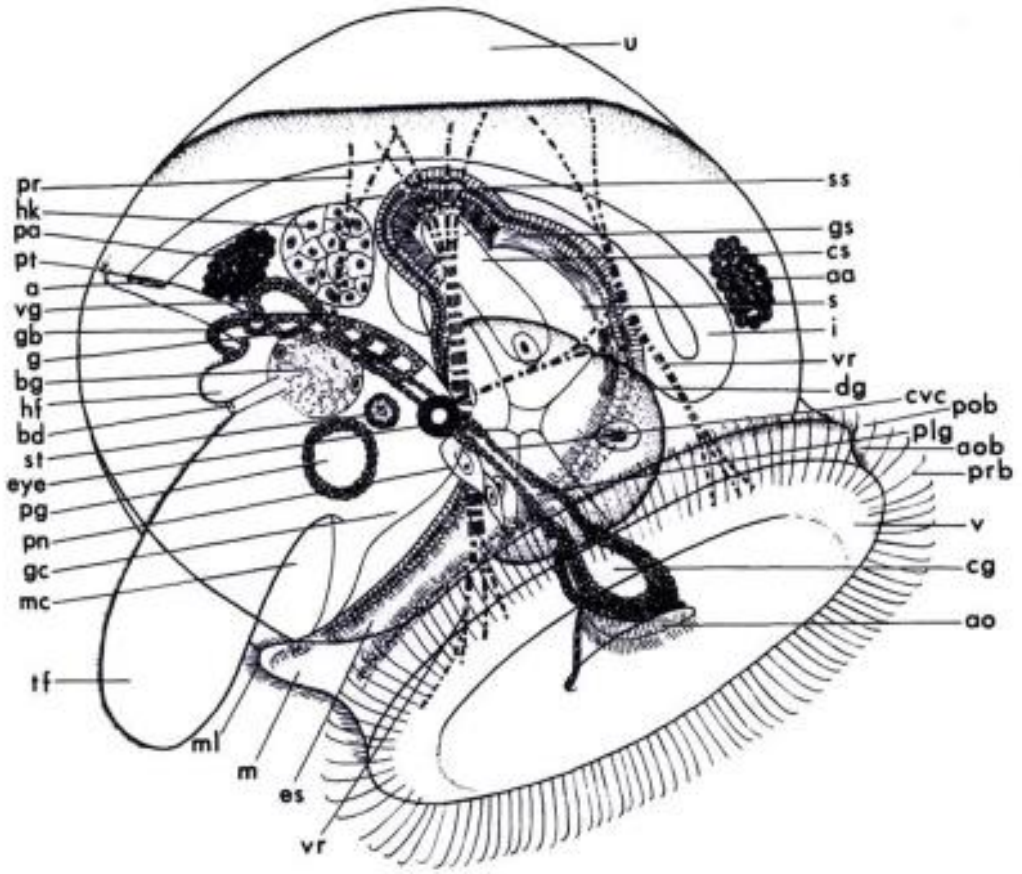

Figure 2.2. Erdmann (1935) diagram as revised by Waller (1981) of the pediveliger stage of oyster *Ostrea edulis.*Abbreviations: a-anus; aa- anterior adductor; ao-apical organ; aob- adoral ciliary band; bd- byssal gland duct; bg- byssal gland; cg- cerebral ganglion; cs- Crystalline sac; cvc- cerebro pleural connective; dg- digestive gland; es- esophagus; eye- eye; g- gill primordium; gb- gill bridge; gc- gill cavity; gs- gastric shield; hf- heel of foot; hk- primordium of heart and kidney; I- intestine; m- mouth; mc- mantle cavity; ml- mouth lobe; pa- posterior adductor; pg- pedal ganglion; plg- pleural ganglion; pn- protonephridium; pob- postoral ciliary band; pr- pedal retractor; prb- preoral ciliary band; pt- post anal ciliary tuft; s- stomach; ss- style sac; st- statocysts; tf- toe of foot; u- umbo; v- velum; vg- visceral ganglion; vr- velar retractor.

Bayne (1971) explained the central nervous system in *Mytilus edulis* as having paired cerebral, pedal and visceral ganglia, but the connectives of these ganglia were not identified in the histological studies. However, he explained the presence of cerebral ganglion lying dorsally to the apical plate. Also at the base of the foot, a pedal ganglia was reported, with visceral ganglia lying posterior of adductor muscle.

Ellis and Kempf (2011) investigated the nervous system in the larvae of the oyster *Crassostrea virginica*. The study suggested that, at D-stage larvae, the apical organ is the only part of the central nervous system. This indicates that there is a considerable neurogenesis yet to be accomplished at this point in development. As the larvae proceeds to pediveliger stage, the presence of an apical ganglion and paired cerebro-pleural, pedal and visceral ganglia has been reported.

Most recently, researchers have developed interest in serotonergic (5-HT), FMRFamidergic, and catecholaminergic (epinephrine, non-epinephrine, and dopamine) neurons of molluscan nervous system. Therefore, these have been extensively studied in gastropods (Dickinson et al., 1999; Dickinson & Croll, 2003; Voronezhskaya et al., 1999; Voronezhskaya et al., 2008) as well as in bivalves (Croll et al., 1997; Voronezhskaya et al., 2008). Immuno-histochemical analyses have described the location and presence of neural compounds including serotonin (5- HT) (Croll et al., 1997; Voronezhskaya et al., 2008), FMRF-amide (Voronezhskaya et al., 2008), acetyl cholinesterase (AChE) activity (Raineri, 1995) and catecholamines norepinephrine, epinephrine and dopamine (Kreiling et al., 2001). These neurotransmitters have received special attention as these are believed to be responsible for the successful transition of larval stage to settlement and metamorphosis (Ellis & Kempf, 2011; Young, 2009). The FMRF-amide neuropeptide was characterised by Price and

Greenberg (1977) for an amino acid sequence. First isolation of FMRF-amide neuropeptide was conducted from the Sunray Venus clam *Macrocallista nimbosa*, and was soon discovered to play an important role in regulating mollusc cardiac muscle (Frontali et al., 1967). This peptide is found across major classes of molluscs, including Bivalvia, Gastropoda, Cephalopoda, Polyplacophora, and Scaphopoda. However, FMRF-amide related peptides are also reported in Insecta, Nematoda and Annelida among Invertebrates as well as in Vertebrata (López-Vera et al., 2008).

The first detailed description of larval neuronal development in general bivalve mollusc has been reported by Voronezhskaya et al. (2008). He investigated the neural development of larval mussel (*Mytilus trossulus*), using FMRF-amide radioactivity. This important study reported the presence of singular FMRF-amide like and serotonin immunoreactive sensory cells, while the larva is still at trochophore stage indicating the beginning of neurogenesis. When the larvae were 2 days old (early veliger stage), an apical neuropil was observed along with the first pedal cell. In 3 dpf (mid veliger stage) larva, a fully developed set of central ganglia pedal, cerebral and visceral ganglia were observed. Finally, interconnecting connectives and commissures were reported extending into each ganglion, when the larva reached pediveliger stage at 22 dpf. This is a common arrangement of ganglia generally seen among the nervous system of many molluscs (Croll & Dickinson, 2004). The study also revealed that after metamorphosis many larval neurons disappear, as do the other larval structures (e.g., prototroch or velum, retractor muscles, etc.) which they innervate. Some neurons in the foot were also observed to disappear while others were retained during metamorphosis. For example, the FMRF-amide labelled sensory cells were absent while catecholamine containing sensory-like neurons were present during late stages of metamorphosis of *Ilyanassa obsolete*.

A better understanding of a bivalve larval nervous system is an important tool for establishing connections between the structure and function of specific sites, as this has been understood through the study of various researchers that the neuronal systems and the neurotransmitters play an important role in settlement (Rodriguez et al., 1993).

2.2.5 Reproduction and larval development of Crassostrea gigas

The general biology of *Crassostrea gigas* have been discussed earlier in detail (Chapter 1). Unfortunately, there is not much literature available on the gametes, fertilisation, and larval development of *Crassostrea gigas*. However, few researchers (Dinamani, 1987; Quayle, 1969a) have recorded reproductive aspects and larval development of the Pacific oyster. Past studies on reproductive physiology suggest that the Pacific oysters are protandrous hermaphrodites (Fretter & Graham, 1964; Katkansky & Sparks, 1966). They can change sex, if conditions compel them to. The adult function as separate male or female animals in reproductive season, but a change of sex occurs from male to female at some point of life. However, Guo et al. (1998) demonstrated that sex in *Crassostrea gigas* is determined by a single gene locus with a dominant maleness (M) allele and an allele for protandric femaleness (F). Sexual maturity is reached during the first year of life (Dinamani, 1987; Nehring, 2006; Pauley et al., 1988).

Under natural conditions, synchronous spawning occurs into the water column, which is attained through mutual stimulation (Galtsoff, 1964). Pacific oysters are highly fecund, as one healthy mature female can produce 50-100 million eggs in a single spawning (Quayle, 1969a). The fertilization membrane forms on the surface of the egg, as soon as sperm enters in it, first maturation division occurs and the first polar body is released After the egg nucleus is fertilised, the egg undergoes cleavage resulting in morula,

blastula and gastrula stages. After completion of embryonic development eggs develop into shelled veliger stage within 48 hours (Park et al., 1989).

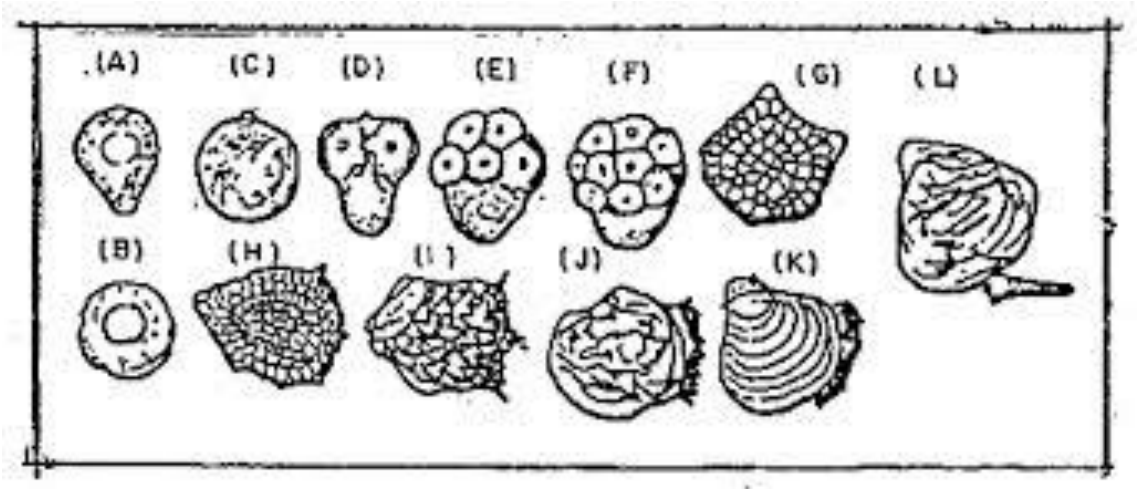

Figure 2.3.Developmental stages of *Crassostrea gigas*. A- egg prior to spawning; B-newly-liberated egg; C- fertilised egg; D- 3-celled stage; E-8-celled stage; F-16-celled stage; G- multi-celled stage; H- ciliated gastrula stage; I- trochophore stage; J- D-shaped veliger larvae; K- early umbonate larvae; L- mature pediveliger larvae (Park et al., 1989).

Table 2.1.Embryonic stages of *Crassostrea gigas* in relation to time at water temperature of 20–21˚C. Modified from Park et al. (1989).

Developmental Stage	Time Required
1st polar body releasing	50 minute - 1Hr 10 min
2nd polar body releasing	1Hr 00min - 1Hr 20 min
1st division	1Hr 20 min - 1Hr 40 min
2nd division	2Hr 00 min - 2Hr 20 min
Morula stage	3Hr 5 min - 3Hr 25 min
Blastula stage	5Hr 20 min - 5Hr 50 min
Gastrula stage	6Hr 10 min - 6Hr 30 min
Trochophore	9Hr 40 min - 10Hr 00 min
D-shaped larva	15Hr 00 min - 28Hr 00 min

Fertilized eggs of *Crassostrea gigas* undergo a series of free-swimming larval stages (trochophore, veliger and pediveliger), over a period of 14 to 22 days. Duration of larvae in each stage depends highly on water temperature (Wallace et al., 2008). For optimal growth water temperatures of 20-22˚C is considered as best (Pauley et al., 1988).

If hatchery conditions are optimal, within 48 hours, 90-95% of the fertilised eggs develops into veliger stage. This veliger stage lasts for 2-3 weeks, during this stage,

larva feeds on phytoplankton caught with help of functioning velum (Pauley et al., 1988). Temperature plays an important role in deciding the duration of each larval stage. For 20-22 days (total rearing period), the optimal temperature (20-22˚C) will enhance the growth of larva with best results (Pauley et al., 1988). Due to high temperature tolerance *Crassostrea gigas* larvae can survive if there are minor temperature fluctuations, but, these fluctuations slow down the larval development resulting in prolonged growth time, hence the risk of predation is increased many folds before the larvae attains sexual maturity (Kennedy & Breisch, 1981). As the larva undergoes further development, eye spot develops in conjunction with foot. This stage is now referred as pediveliger stage. When the larva attains suitable size its foot protrudes out and it starts looking for suitable substratum to attach (Pauley et al., 1988). As soon as it finds a suitable substratum, larva attaches itself and now it is called spat. Other factors which plays vital role in deciding the venue of attachment are the presence of other oysters, sufficient light and surface irregularities (Pauley et al., 1988). As the larva undergoes metamorphosis, some important morphological changes occur such as loss of functional foot and velum and development of gills (Loosanoff, 1965; Quayle, 1969a). The shape of oyster shell greatly depends upon the type of substratum it attaches itself. If they grow in sand , they attain longer shell length, but are narrow in diameter, if they are attached to hard surface like gravel, they have irregular surface and are deeply cupped (Quayle, 1969a). Finally, the larva spends rest of its life while attaching to the substratum and leads a sessile life (Pauley et al., 1988).

2.2.6 Significance of research on Crassostrea gigas larvae

Crassostrea gigas is one of the most important oyster species due to its high, recreational (cultural harvesting), economical and environmental significance (Bell, 2005). This species alone is a huge source of revenue globally due to its high nutritional value. High fecundity of *Crassostrea gigas* larvae makes it readily accessible for study. Vast amount of literature present for this species indicate the interest of researchers. This species shares common larval development characteristics with other important commercial species, such as mussels which are being extensively investigated for their commercial importance, hence any new outcome will help in conservation of all the relevant species. Being a filter feeder *Crassostrea gigas* can be used as a biological indicator of all the estuarine water qualities and introduction of this species can greatly help to improve the water quality. This oyster is sometimes referred to as nature's engineers. Collectively all the above features makes *Crassostrea gigas* larvae an ideal material for investigation and it is suggested that identification and characterisation of all biological aspects of *Crassostrea gigas* will help in future research and hatchery cultivation.

2.3 Aims and objectives

The aim of this chapter is to describe the larval development of *Crassostrea gigas* from day 1 through to post-settlement.

The objectives are

- To describe shell morphology of larvae through scanning electron microscopy, and light microscopy.
- To describe survivability and feeding patterns of developing larvae over the total rearing period of 22 days.
- To describe organogenesis processes through light microscopy.
- To describe neurogenesis through immunochemistry.

2.4 Materials and methods

2.4.1 Broodstock

Mature broodstock of the Pacific oysters (*Crassostrea gigas*) were supplied by Pacific Marine Farms from Northland, New Zealand. Broodstock were used immediately upon arrival at Glenhaven Aquaculture Ltd, Cawthron Institute in Nelson. Firstly, these broodstock were cleaned carefully to remove fouling organisms, then the oysters were carefully shucked open and a small sample of gonad taken and examined microscopically to determine sex. Females were then stripped by lacerating the gonad and then oocytes were washed gently with the Pasteur pipette into a beaker containing 1µm filtered UV treated seawater (SW). The seawater containing oocytes was then passed through a 62µm mesh screen to remove any debris. Sperm were collected as dry as possible by lacerating and pressing the male gonad with the Pasteur pipette and allowing the contents to drip into a 70ml plastic container. A small amount of seawater (1-2ml) was washed over the gonad to encourage dripping. Three pools of oocytes were made by combining oocytes from 3-4 individuals per pool. Oocyte concentration was determined by counting replicate aliquots of oocytes of a known dilution. Sperm concentration was determined by using a Neubauer haemocytometer. Sperm were pooled from three individuals to fertilise the oocytes. For fertilization, three replicates of 20 million oocytes per pod were fertilised at a density of 1000 oocytes ml^{-1} and a sperm concentration of 200 sperm per oocyte were used. After a contact period of 20 min, oocytes were transferred to tanks containing 150 L of SW and 4 µM EDTA (10 million per tank) to remove any heavy metal like zinc or lead not removed through filtration, and left to incubate at 23˚C for about 22 h until they reached the D-larval stage.

D-larvae from each tank were drained onto a 45µm mesh screen and collected as concentrated as possible. Larvae from three replicate incubation tanks per pool were combined for each and concentrated into 10ml tubes. The concentration of larvae was 3.2 - 4.1 x 10^6 ml^{-1}. Concentrated larvae were then divided into 1ml aliquots and diluted 1:1 with seawater and loaded into straws at room temperature (23˚C). These larvae were being used as control on a cryopreservation study (Chapter 3) this is the reason why they were loaded into the straws. Two straws for each of the three replicate control tanks were taken. The contents of each straw were then added into 1ml of FSW (Fresh sea water) containing 0.1% BSA (Albumax®I, Gibco®, Life Technologies, New Zealand). A total of 500,000 larvae per replicate control tank were then transferred to specialised rearing tanks called Cawthron Ultra Density Larval Rearing System (CUDLS) (King et al., 2005; Ragg et al., 2010).

2.4.2 General larval rearing

Larvae were reared in 2.5 L bullet shaped tanks in the CUDLS. Briefly, the system provides a constant environment to the larvae with continuous SW, microalgae and air supplied to each tank. Two glass rods supply the tank with SW, microalgae and air, and a saber screen retains the larvae in the tank. Larvae were fed on a mixed diet of 1:1 *Isochryis galbana* and *Chaetoceros calcitrans* at a rate of 80-180 cell μL^{-1}. Seawater was supplied to the tanks at the rate of $80ml^{-1}$ for days 1-9 of larval rearing and at 120 ml min^{-1} thereafter. Saber screens were cleaned daily with hot fresh water and every second day larvae were screened on increasing mesh sizes in accordance with standard hatchery practices to eliminate dead and decaying larvae. During screening, tanks, saber sieves and glass rods were washed down thoroughly with hot fresh water. Larvae were resuspended in a 1L beaker containing SW using a large diameter, perforated plastic

plunger and samples were taken for microscopy, growth measurements and survival. Remaining larvae were then returned to the tank for further rearing.

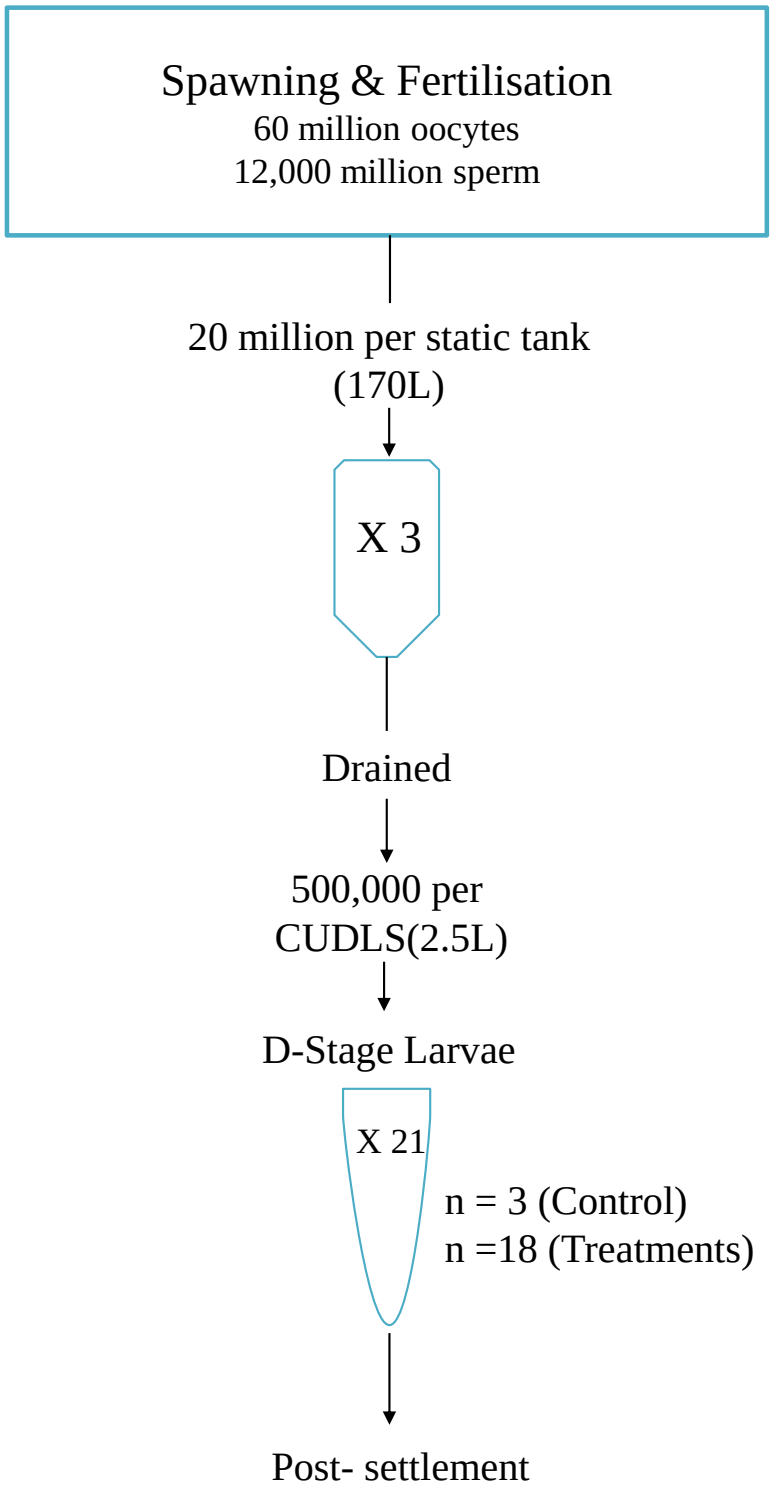

Figure 2.4.Experimental design showing larval rearing from fertilisation to post-settlement.

2.4.3 Survivability

The survivability for each CUDLS was determined on each alternate days 3, 5, 7, 9, 11, 13, 15, 17, 19, 21and 22. The tanks containing larvae were drained onto a 45µm screen and then these were gently washed into a 1L beaker with FSW. Three replicate samples of 200µL were taken and fixed using lugols iodine. The number of larvae in each sample was counted using an inverted microscope (Olympus CK2 inverted microscope) to estimate the actual number in each replicate. Daily sampling for Light microscopy, SEM, and confocal microscopy were extracted into eppendorfs for later observations, when working out the overall remaining population density.

2.4.4 Shell length

Every alternate day, 200 µL of water subsample was fixed with lugols iodine from each CUDLS to view the larvae. Olympus CK 2 inverted microscope with a mounted Olympus C-7070 7.1 mp camera at 20x objective was used to take photomicrographs for each sample. The longest measurement across the ovoid of the shell referred to as Feret's diameter was measured using customised particle recognition macro in a java based image processing software ImageJ.

2.4.5 Feeding consumption

A hand held fluorometer (Cyclops-7 chlorophyll - a fluorometer with a 30 mL black polyethylene sample chamber) was used to determine the larval feeding consumption of mixed microalgal diets, composed of *Isochrysis galbana* and *Chaetoceros calcitrans.* These readings were taken every alternate day for each replicate CUDLS and were

recorded millivolts (mV). The following equation A was used to determine the consumption rate per tank.

Equation A:

% Consumption = $\Delta mV / (mV_{in} - mV_{blank}) * 100$.

Where:

mVin = inflow reading

mVout = outflow reading

ΔmV = difference between mVin and mVout.

mVblank = mV reading before the introduction of diet.

2.4.6 Light microscopy

Light microscopy pictures were taken using An Olympus CK2 inverted microscope with a mounted Olympus C- 7070 7.1 mp camera with 20x objective was used for the visual assessments of oyster larvae development for each alternate day. Multiple pictures of each sample were taken at same position but at different focus. These images were stored in JPEG format and later these images were stacked using stacking software CombineZP (Combine Z-pyramid). Images were later modified for their brightness and contrast using Adobe Photoshop CS5.The progression of organogenesis was observed by taking photographs of morphological and physiological changes. The onset of settlement clues, such as eye spot, reabsorption of velum and development of gill

rudiment were also observed. Behavioural observations, such as movement of velum and foot were taken to assess the viability of larvae.

2.4.7 Scanning electron microscopy

A sample of around 500 control larvae of the Pacific oysters were taken from three replicate static tanks system every alternate day from the first day until post-settlement day from CUDLS. After pipetting out these larvae from respective tanks, they were gentle placed onto a 43 µm mesh. Using a disposable eye dropper, samples were carefully transferred into a 1.5 ml eppendorph tube containing ¼ strength seawater. Precautions were taken to prevent rupturing of the shells as these are very delicate at this stage. Filtered seawater (1µm) containing 7.5% $MgCl_2$ was then added drop wise to each eppendorf to relax the larvae, until the larvae were immobile and concentrated on the bottom of the tube. Excess seawater was then removed and larvae were fixed in 10% Formalin (in FSW) for 24 hours at room temperature. Larvae were then washed in PBS (Phosphate buffered saline) and placed in 70% ethanol for storage at -20˚C freezer. For SEM analyses larvae samples were taken out of the -20˚C freezer a day before preparation and transferred to a 4˚C environment. Larvae were then centrifuged at 2,000 x g (Bio Rad centrifuge, Korea) for 30 sec to concentrate them at the bottom of the tube. A 70 % ethanol wash was performed, followed by dehydration with an ascending series of 1 wash of 90 % ethanol followed by 2 washes of 100 % ethanol at 15 min intervals. Larvae were gently placed onto SEM stubs and placed in a desiccator for 48 hours for drying. The specimens were then sputter coated with gold, a few hours before observation with a Hitachi SU-70 Shottky field emission SEM microscope. The sputtering is done with great care as over-exposure or under-exposure affects the quality

of images. The images were later adjusted for brightness and contrast as well as the background was filled in black using Abode Photoshop CS5.

2.4.8 Confocal microscopy

A sample of around 500 control veliger larvae of the Pacific oysters were taken from three replicate static tanks system every alternate day from the first day until post-settlement day from CUDLS. These larvae were gently siphoned out using a disposable eye dropper and placed into a 1.5 ml eppendorph tube containing ¼ strength seawater. Once relaxed with $MgCl_2$, larvae were fixed in 10% formalin for 24 hours within 10 minutes so that larvae were immobilised to make it easier to identify the various neurons during observation under the confocal microscope. Then, the larvae were stored in 70% ethanol at -20˚C. For confocal analyses, larvae samples were taken out of the -20˚C freezer a day before preparation and transferred to a 4˚C environment. Larvae were then centrifuged at 2,000 x g (Bio Rad centrifuge, Korea) for 30 sec to concentrate them at the bottom of the tube. After removing excess ethanol, larvae were transferred to a BD Falcon low evaporation 96 well plate. Larvae were then processed using a modified procedure from Voronezhskaya et al. (2008). Larvae were then rinsed three times in 100µl PBS for 15 mins. The shells of larvae were then decalcified in 5% EDTA in 100µl PBS for 6 hours to ensure complete decalcification, then rinsed in 100µl PBS for 15 mins. The samples were blocked in 100µl PBS in 4 degrees on a gentle agitator overnight, containing 10% normal goat serum, 0 25% bovine serum albumin, 1% Triton X-100 (TX), and 0.03% sodium azide.. Larvae were incubated with FRMF amide AB (diluted 1:500 in PBS with 1.0% normal goat serum and 1.0% TX) for 72 hours at 4˚C with gentle agitation. This step was skipped for control larvae to avoid binding with primary antibody and to determine the presence of FMRFamide immunoreactivity. The

larvae were then given 3 washes (3 x 1 hour) of 100µl PBS and incubated for 4 days in goat anti-rabbit IgG (Alexa 488) and diluted in solution of 1:50 in PBS at 4˚C with slow agitation. This step and following steps were performed in dark to avoid photo-bleaching of the samples. Larvae were rinsed several times in 100µl PBS after incubation in the secondary antibody. Samples were gently transferred to a µ-slide 8 well ibitreat chamber with AF1- Citifluor mountant. Control without a primary antibody was used to distinguish between positive and negative neuropeptide cell responses.

Finally, all the larvae were examined under a Zeiss LSM 710 Inverted confocal microscope with 20X objectives. Appropriate wavelength filter settings were used when required. ZEN 2009 Light edition was used to obtain three dimensional rotatable images, which were later converted into Z stacked LSM files and finally into single projection, which were exported as TIFF images. These images were processed with Adobe Photoshop CS 5 to adjust for brightness and contrast.

2.4.9 Preparation of solutions for confocal microscopy

Experimental reagents:

10% Normal Goat serum and Alexa Flour® 488 Goat Anti-Rabbit IgG were supplied by Invitrogen Life Technologies, Auckland. FMRF-amide (Cardio-excitatory Peptide) Antibody was supplied by Immunostar (Hudson).

Preparation of solutions:

Standard PBS solution was prepared by adding 8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4 and 0.24g of KH2PO4 in 800 ml of distilled water. Required pH was then adjusted by adding HCl and NaCl, further more distilled water was added to make a total volume of 1L.

Decalcification solution (5% EDTA) was prepared by adding 5g EDTA in 100ml of stock PBS solution. For the preparation of blocking solution, 10ml of goat serum, 0.25g of bovine serum albumin, 1ml of Triton-X 100 and finally 0.03g of Sodium azide was added to 88.75ml of PBS stock solution. Next step of preparing primary FMRF amide antibody solution was conducted by adding 0.5ml of goat serum, 0.5ml of Triton-X 100 and finally 0.1ml of FMRF antibody in 48.9ml of PBS stock solution to get an overall dilution of 1:500. Finally Alexa 488 secondary antibody was prepared by adding 0.5ml of Alexa fluor 488 in 24.5ml of PBS stock solution to get desired dilution of 1:50.

2.4.10 Statistical analyses

To analyse the data obtained from experimental results, Statistical 6 software was used. A repeated measure ANOVA was used to analyse the survivability data. These data did not need transformation to meet statistical requirements. A repeated measure ANOVA with Tukeys tests was done to analyse the shell length data.

2.5 Results

2.5.1 Survivability analyses

Survivability in the CUDLS showed a significant decrease during the 22 days rearing period (ANOVA; $F_{10,22}$ =27.86, $p < 0.05$. Survivability % decreased sharply over the first 5 days of total larval rearing period. This reduction in survivability reaches to as low as 30% by day 7 and after that these levels were maintained until day 19. A sharp decline was observed after day 19 and only 15,533 ± 613 larvae suvived to day 22. On day 19, larvae were accidently exposed to a higher dose of UV than normal, which may have contributed to the increased mortality between day 19 and day 22.

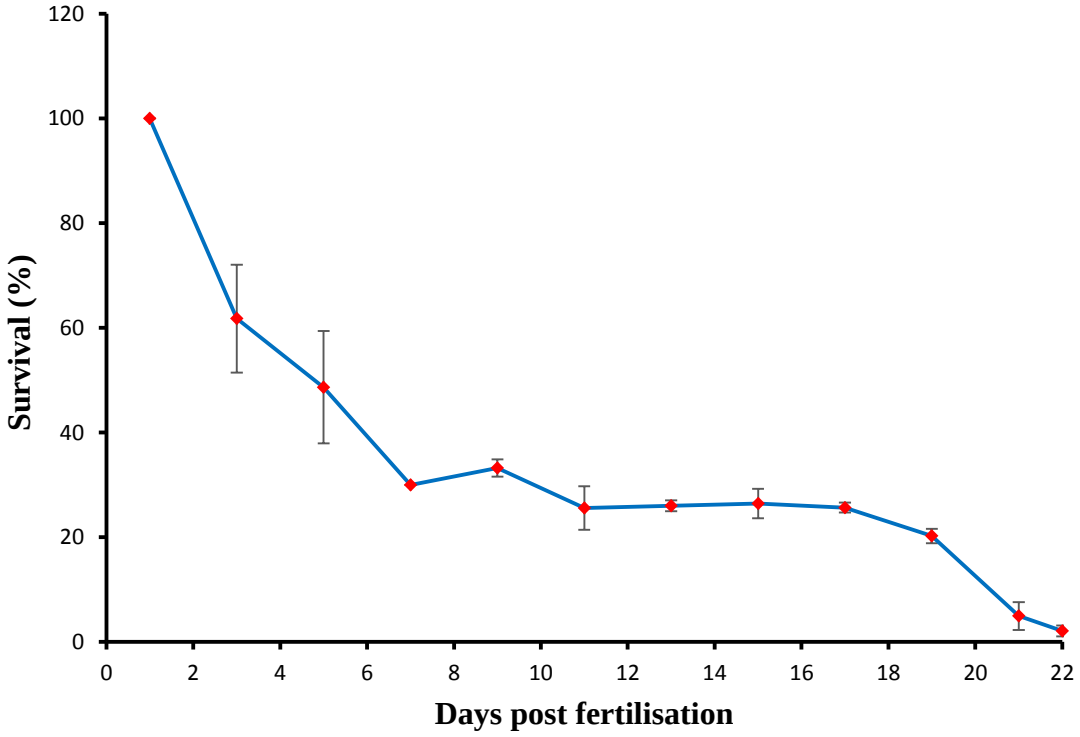

Figure 2.5.Average percent survivability (± SE) of *Crassostrea gigas* larvae over 22 days rearing period.

2.5.2 Shell length analyses

A strong correlation (R^2= 0.8373 between shell length (µm) and larval age (days) was observed. The average growth of shell length per day was found to be 10.68-µm per day which suggests positive linearity between shell length and the age of larvae (ANOVA; $F_{10, 22} = 2.92$, $p < 0.05$). The initial shell length on day 1 was found to be 79.0 ± 2.3 µm with a final shell length of 313.8 ± 14.2 µm on day 22. However, from day 17 onwards a stable trend was observed, with not much increase in shell length, which suggests that larvae were using their metabolic energy in preparing themselves for settlement process.

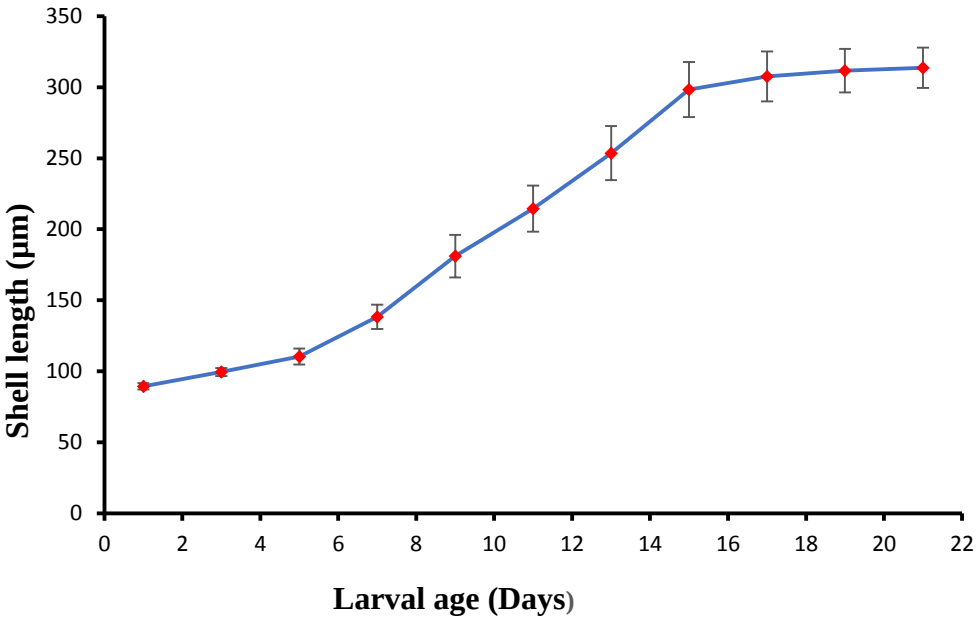

Figure 2.6.Average shell length (µm ± SE) of *Crassostrea gigas* larvae over 22 days rearing period.

2.5.3 Feeding consumption analyses

Feeding consumption showed an increasing trend for the first 7 days, with a sharp increase on day 5 and then a subsequent decrease on day 9 (Fig 2.7). Two subsequent consumption peaks were observed on day 11 and day 17, which had 39.5 ± 7.6 mV day^{-1} and 40.5 ± 5.2 mV day^{-1}, respectively. By day 19 feeding consumption was less than 10mV day-1. This fall can be accounted to high mortality rates and preparation of larvae to undergo metamorphosis, between day 19 and 22. Feeding consumption over the total 22 days of feeding was statistically very significant (ANOVA; $F_{10, 22} = 4.43$, $p < 0.05$).

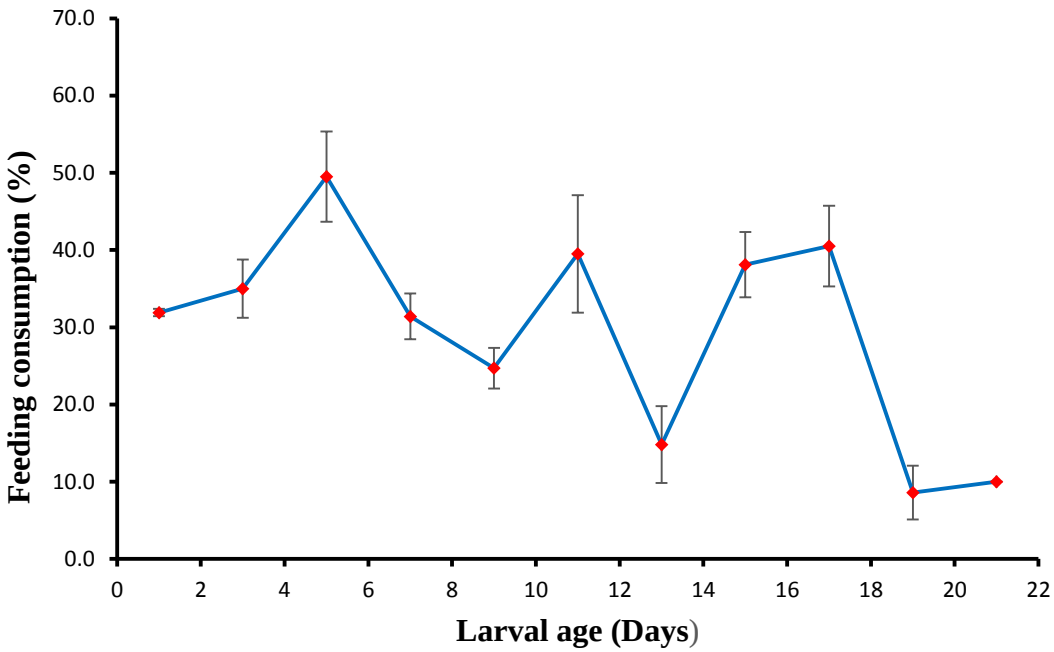

Figure 2.7.Average percent feeding consumption (± SE) of *Crassostrea gigas* larvae over 22 days rearing period.

2.5.4 Shell morphology

Study of development of the Pacific oyster covers the development of D-stage larvae from day 1 through to post settlement. D-stage larva (1 day old) with an average shell length of 79.0 ± 2.3 µm showed a PI layer with a prominent pitted punctate pattern and a stellate radial region extending from the margin to the edge of the mantle and hinge (Fig. 2.8A). As the larva progressed towards the day 5, their shape changed from D-shape to more or less round shape with appearance of PII layer showing comarginal growth rings (Fig. 2.8B). Anal tuft was observed protruding out at the base of shell. The velum had developed to aid feeding. Another day 5 larval specimen at higher magnification showed many singular cilia protruding out (Fig 2.9). Progressing towards day 9, larvae reached an early umbo stage with an average shell length of 181.04 ± 14.92 µm. Larvae displayed a developing umbo and further secretion of PII protruding outwards towards the mantle edge (Fig 2.8C). Late umbonate larva at day 15 displayed a well-developed umbo and the concave shape of upper (left) shell can be clearly noticed. The umbone of the left valve is much larger than the right valve, creating unequivalant condition (Fig- 2.8D). Umbonate larva (17 days old) with an average shell length of 307.66 ± 17.56 µm possess a well-developed umbo and the rounded shell appearance. Postero dorsal notch extending from the margin of the PI to the external edge was observed (Fig.2.8E). Another day 17 showed postero dorsal notch extending from the margin of the PI to the external edge with higher magnification (Fig 2.11). The two valves of the shell fit tightly to each other except at this point, at the notch, the edge is either absent or is folded back, this arrangement provides a tight seal to the shell but prevent sheer between the valves. As the larva progressed towards post larval stage (21 days old) with the shell length of 313.78 ± 14.18 µm, the velum was totally reabsorbed

and the presence of a dissoconch layer forming at the mantle edges indicated the onset of settlement transition (Fig 2.8F).

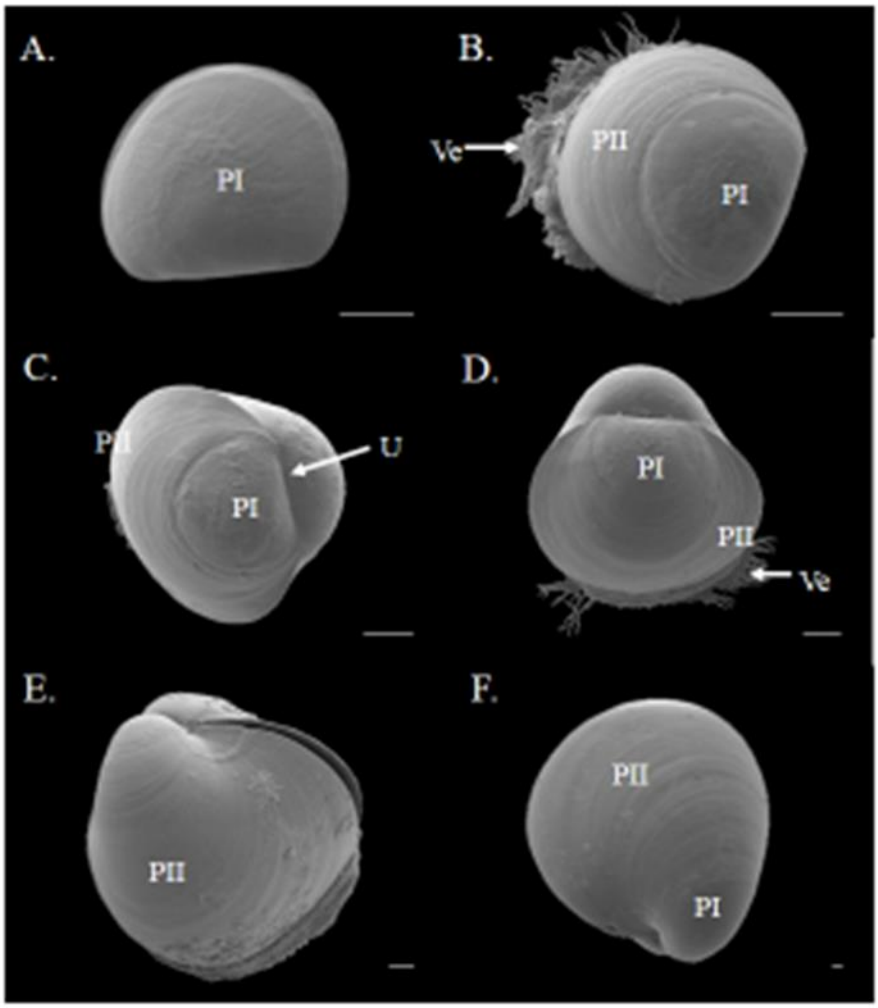

Figure 2.8.Figs A-F. Shell development of *Crassostrea gigas*. A- D-stage larva (1 day old); B- D stage larva (5 days old); C- Early umbo stage (9 days old); D- Late umbonate stage (15 days old); E-Umbonate

larva (17 days old); F- Post larva stage (21days old). Abbreviations: PI- prodissoconch I; PII- prodissoconch II; Sr- stellate-radial region; U- umbo; Ve- velum. Scale bar = 20µm.

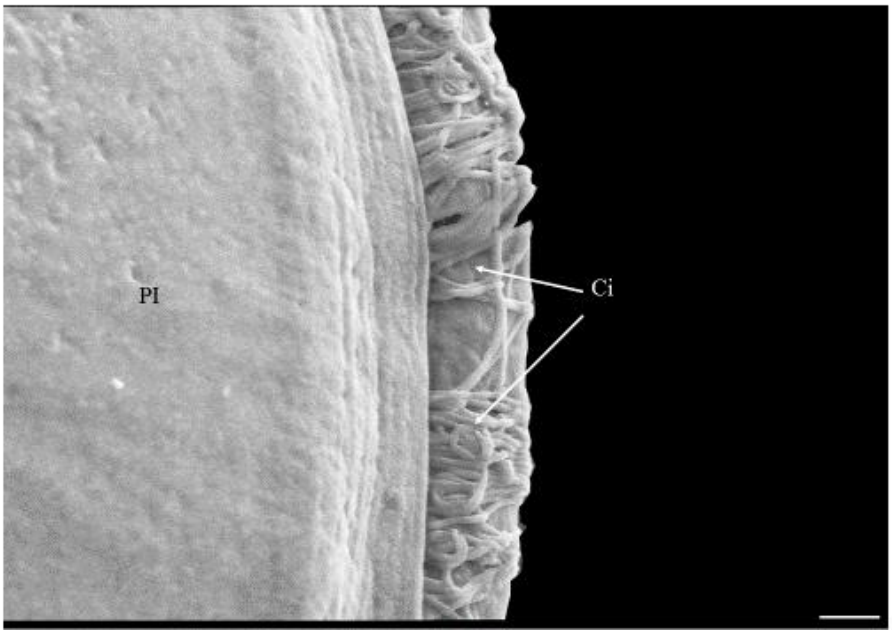

Figure 2.9.SEM picture of 1 dpf larva at higher magnification showing cilia. Abbreviations: PI- prodissoconch I, Ci-cilia. Scale bar = 2 µm.

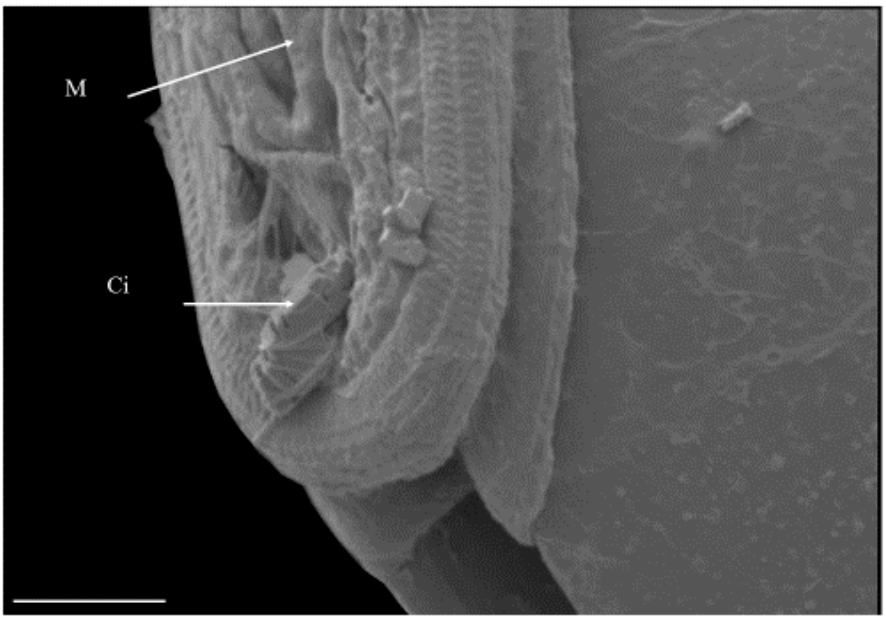

Figure 2.10.SEM picture of a 15 dpf larvae showing different whorls of velum around the mouth. Abbreviations: M-mouth; Ci-cilia. Scale bar = 20 µm.

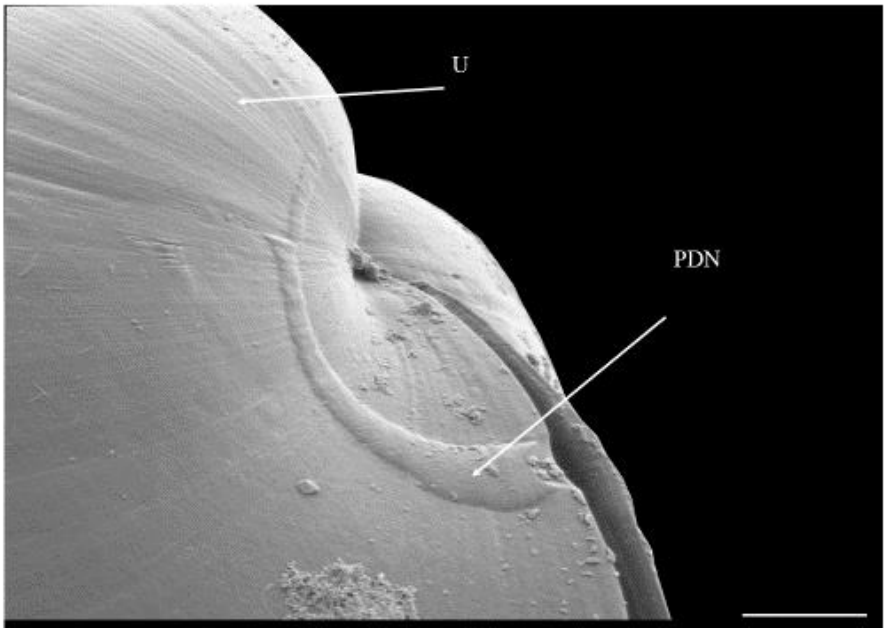

Figure 2.11.SEM picture of a 17 dpf larvae showing postero dorsal notch. Abbreviations: U- umbo; PDN- postero dorsal notch. Scale bar = 20 µm.

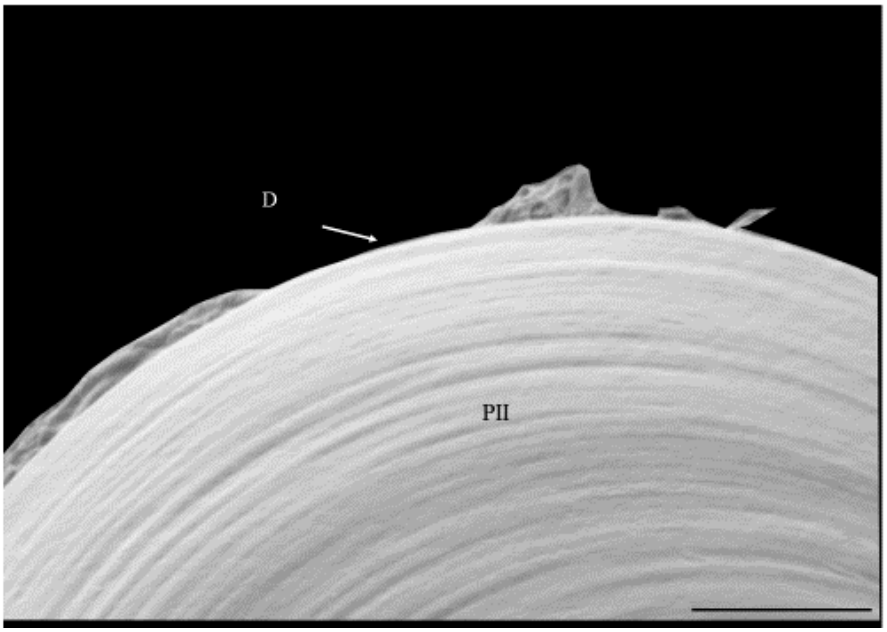

Figure 2.12.SEM picture of a 21 dpf larvae showing dissoconch layer. Abbreviations: D- dissoconch; PII- prodissoconch II. Scale bar = 20µm.

2.5.5 Organogenesis

Complete organogenesis was observed from day 1 through to settlement. Light microscopy revealed a very limited amount of organogenesis at day 1 with only digestive diverticulum and numerous cilia on hinge being visible. The larvae were typical D-shaped with a straight hinge and the beginning of stomach is visible (Fig- 2.13A). At day 3, a well-developed velum was seen extending outwards of the valves. A close observation revealed beating of the cilia, which helps larvae in locomotion and feeding. A developing alimentary canal was observed with a style sac originated at the dorsal end of the stomach near the hinge, along with digestive diverticulum and the velar retractor muscle started developing, which are attached to the velum and are anchored at the opposite end of the shell (Fig- 2.13B). The digestive diverticular aids in digestion. It is made up of a number of blind tubules, which empty into large ducts which lead to the interior of the stomach.

As the larvae progressed to day 5, the shape changes from typical D to an oval shaped early umbo stage. The umbo was seen developing on the dorsal side. Organogenesis had progressed well with a well-developed mouth, esophagus, stomach and digestive diverticulum, (Fig- 2.13C). A velum with numerous well beating cilia was observed. Another photograph (Fig- 2.14) shows a group of larvae at day 5 assuming more or less a round shape with a developing umbo. At day 13, well-developed umbones were observed. Digestive diverticulum showed well-defined lobes in which a great amount of microalgal accumulation was seen. Other internal organs could not be observed because of high algal content (Fig- 2.13D). As the larvae proceeded to day 15 along with the velar retractor muscles, well developed anterior adductor muscle can be seen, the gill rudiment was seen emerging, consisting of short tubular channels (Fig 2.15).

At day 17 (pediveliger stage), a highly contractile velum surrounded by tactile cilia was observed. A relatively large esophagus leading to a barrel like stomach which is in close contact with the glandular structure of digestive diverticulum was seen. The intestine was seen emerging from the stomach, making a single loop and continues into the rectum. An anus opening was also observed (Fig-2.13E). In Fig - 2.16, pseudofeces can be seen emerging out of the larval shell. This pseudofeces elimination is specialised final rejection step in the cascade of particle processing events which begin with capture on the gill. A gill rudiment was observed as the series of short twisted tubular channels. The heart and kidney rudiment was also seen as a group of cells at the base of digestive diverticulum. At day 19, a highly ciliated foot was observed in one of the larva (Fig – 2.17). These cilia are thought to be highly sensitive, bearing statocysts, which helps the larvae to find a suitable substratum and finally it is destined to degenerate. At the base of foot lies byssal gland, which secretes cement like substance that binds larvae to the substratum.

At day 21, the larvae showed all the signs of successful metamorphosis and were characterised by the presence of an enlarged digestive diverticulum, gill rudiment, adductor muscle and functional foot. The foot was seen extending out in some larvae. The velum was starting to reabsorb as the larvae prepared to settle (Fig 2.13F). Visual observations identified eye spots in most of the larvae. At day 22, (Fig 2.18) the velum was completely withdrawn in the shell cavity as can be seen in the figure. At this stage the valves closed and the larvae dropped to the bottom. At this contracted stage, all the other organs have become undistinguishable. The foot has degenerated as the larvae settles down and acquires a sedentary mode of life.

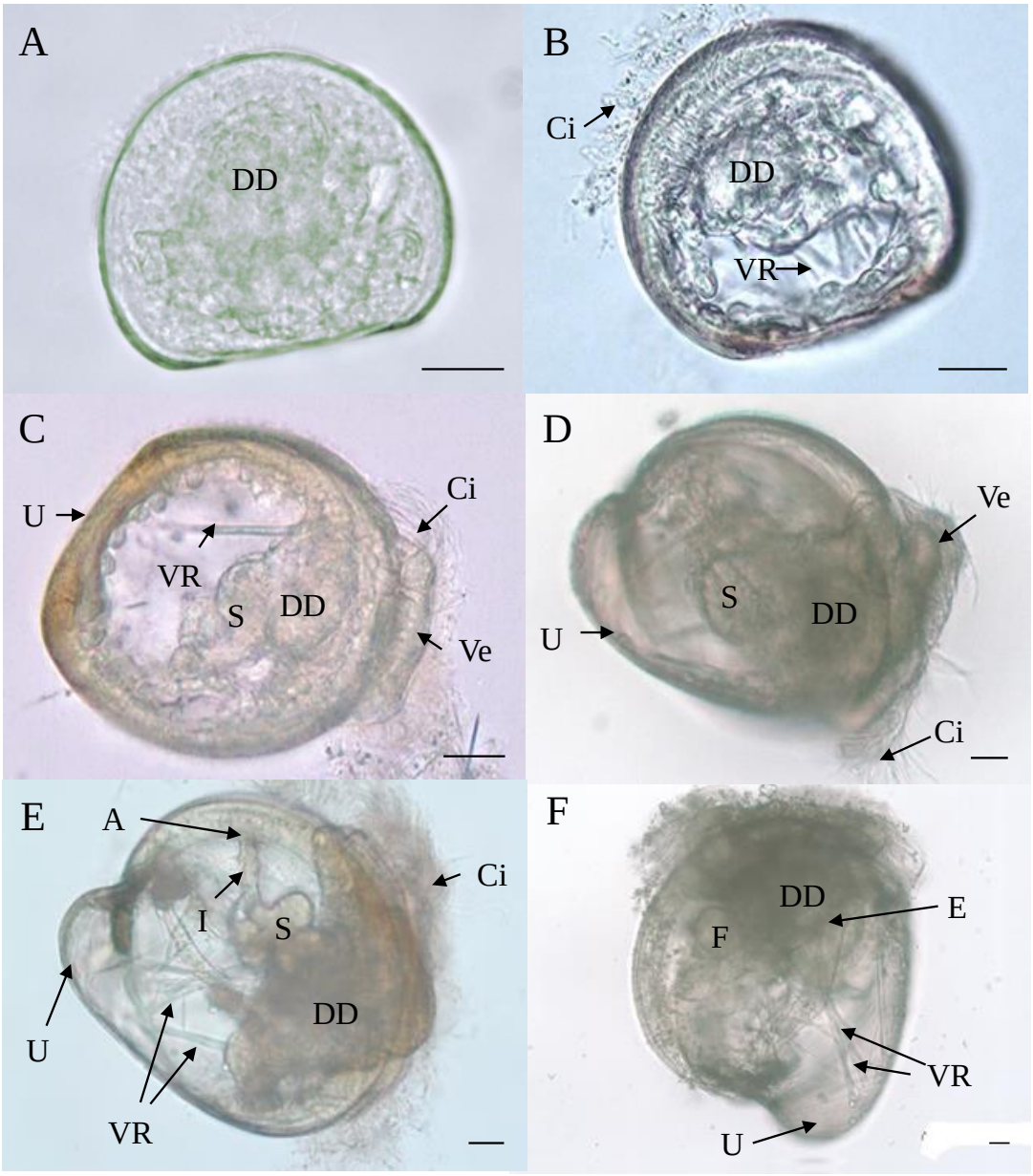

Figure 2.13.Fig A-F. Light microscopy images of *Crassostrea gigas* showing overall organogenesis and microalgal content in the stomach. A- D-stage larva (1 day old); B- D stage larva (3 days old); C- Early umbo stage (5 days old); D- Late umbonate stage (13 days old); E- Umbonate larva (17 days old); F- Post larva stage (21 days old). Abbreviations: A- Anus; Ci- Cilia; DD- Digestive diverticulum; E- Esophagus; F- Foot; I- intestine; S- Stomach; SS- Style sac; U- Umbo; Ve- Velum; Vr- Velar retractor muscle. Scale Bars = 20µm.

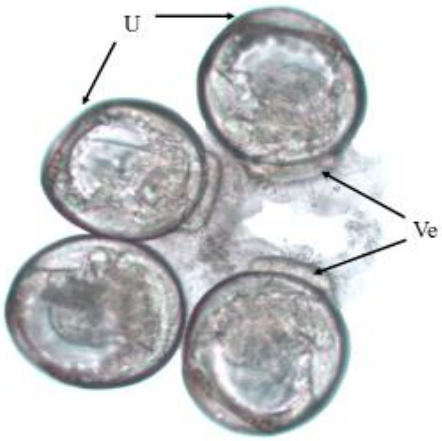

Figure 2.14.Light microscopy photograph of *Crassostrea gigas* 5 dpf larva showing developing umbo and velum. Abbreviations: U- Umbo; Ve- Velum. Scale bar= 20µm.

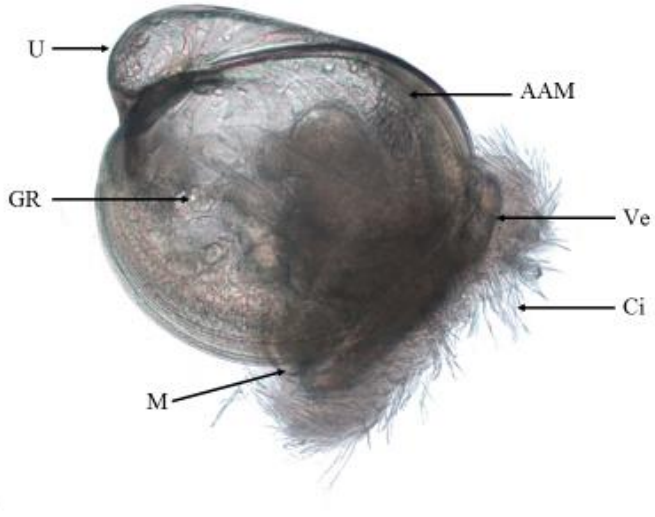

Figure 2.15.Light microscopy photograph of *Crassostrea gigas* 15 dpf larva. Abbreviations: AAM- anterior abductor muscle; Ci- cilia; GR- gill rudiment; M- mouth, U- umbo; Ve- velum. Scale bar= 20µm.

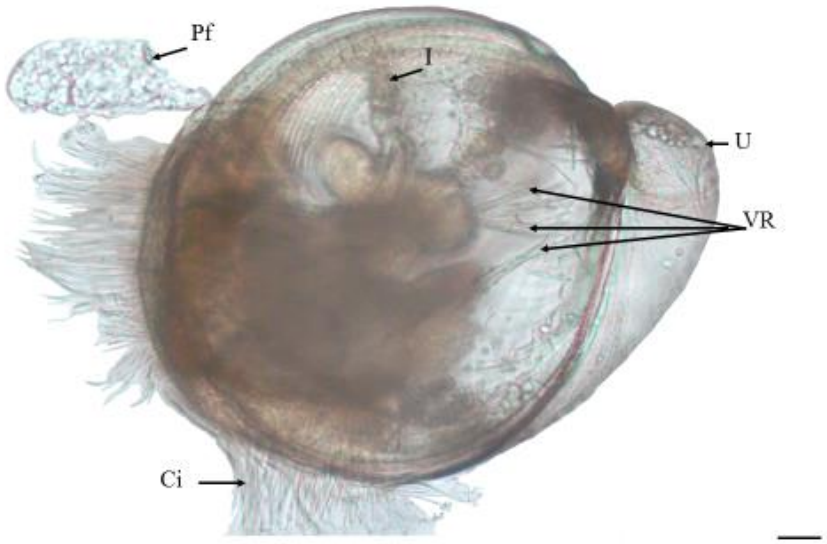

Figure 2.16.Light microscopy photograph of *Crassostrea gigas* larvae 17 dpf releasing pseudofeces. Abbreviations: Ci- cilia; M- mouth; P- pseudofeces; U- umbo; Ve- velum. Scale bar = 20µm.

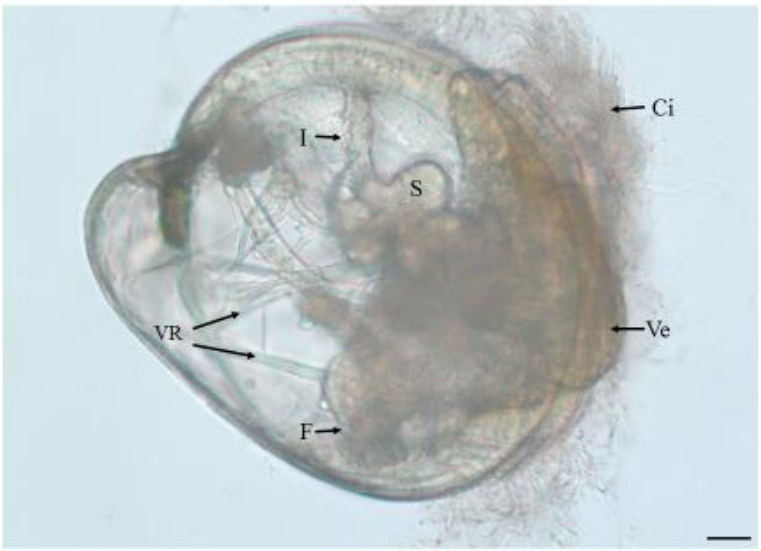

Figure 2.17.Light microscopy photograph of another *Crassostrea gigas* larvae 17 dpf showing highly ciliated foot. Abbreviations: Ci- cilia; F-foot; I-intestine; S-stomach; Ve- velum; VR- velum retractor muscles. Scale bar= 20µm.

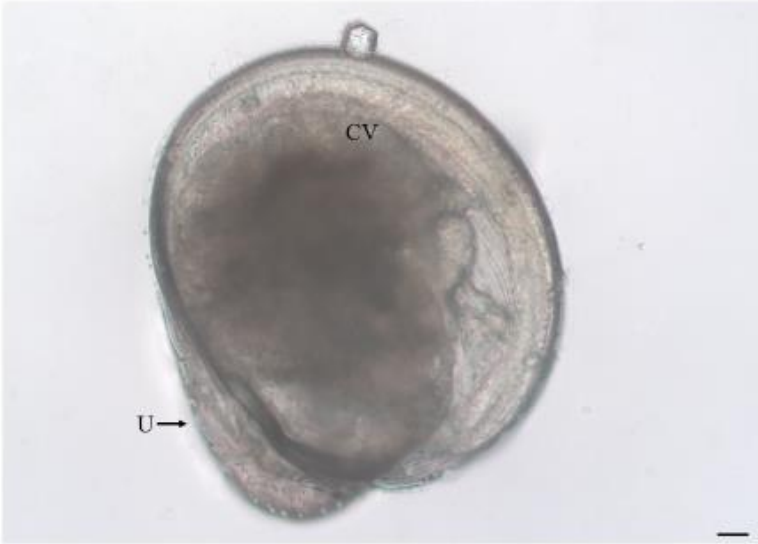

Figure 2.18.Light microscopy photograph of *Crassostrea gigas* larvae 22 dpf showing contracted velum. Abbreviations: CV- contracted velum; U- umbo. Scale bar= 20µm.

2.5.6 Neurogenesis

An effort to study the process of neurogenesis, along the development of *Crassostrea gigas* larvae did not give satisfactory results as the larvae were unable to absorb sufficient amount of florescence. However, the results found are explained below. At 1 dpf, the D-shaped larva possess very little FMRFamide- like radioactivity. Radioactivity was observed on the mantle suggested to be mantle neuron. Sections of digestive diverticulum also showed some immunoradioactivity. There is origin of the apical/cerebral ganglion, but because of poor absorption of FMRFamide by the larvae, it is not very clear in the pic (Fig 2.19). At 7 dpf, the mantle nerve is brightly stained, velum is rich in immunoreactivity as the cilia are highly innervated by neurons. The mantle nerve is continued into the mantle cavity. Again, due to poor absorption of FMRFamide by the cells, other neuronal structures were not clear in the pictures. Hence, no descriptions can be provided in detail (Fig 2.20). At 21 dpf, the cerebral

ganglion should be deeply stained, indicating the neuronal development along with the peripheral nervous system, but due to lack of absorption of FMRF amide, it is not clear at all, and neither are the other nerves and ganglion. However, highly innervated foot was seen (Fig. 2.21), which indicates that the foot is rich in sensory neurons and now plays important role helping the larva find a suitable substratum to attach itself and undergo successful metamorphosis.

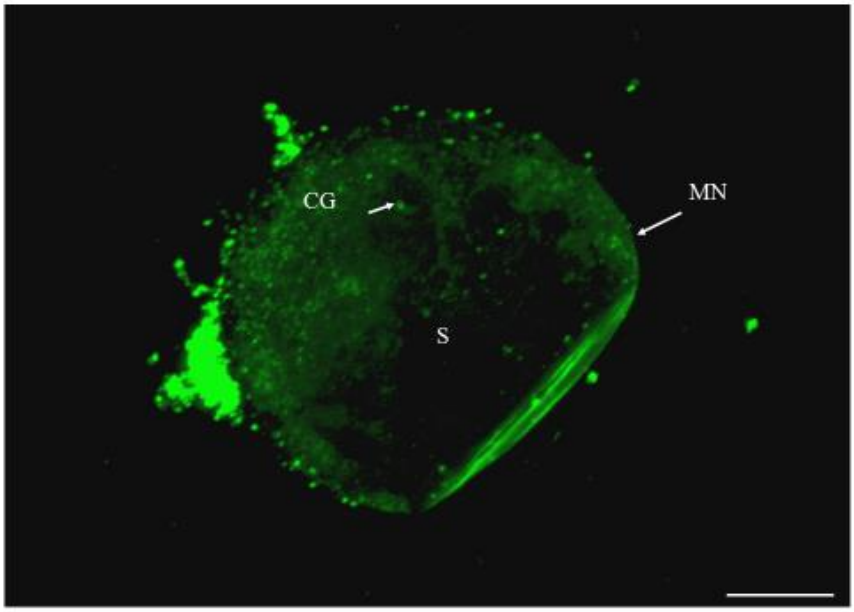

Figure 2.19.FMRFamide-like green immunoreactivity in a 1 dpf D-stage larva. Abbreviations: CG- cerebral ganglion; MN- mantle nerve; S- stomach. Scale bar = 20µm.

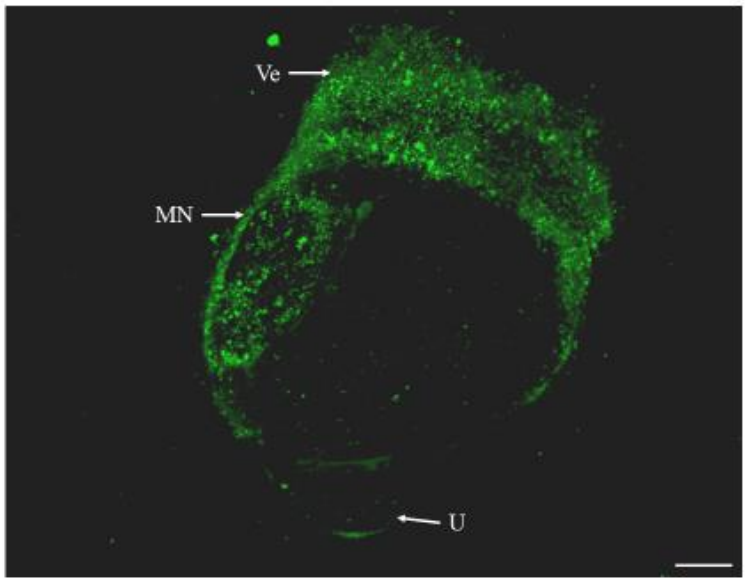

Figure 2.20.FMRFamide-like green immunoreactivity in a 7 dpf larva. Abbreviations: MN- mantle nerve; U- umbo; Ve- velum. Scale bar = 20µm.

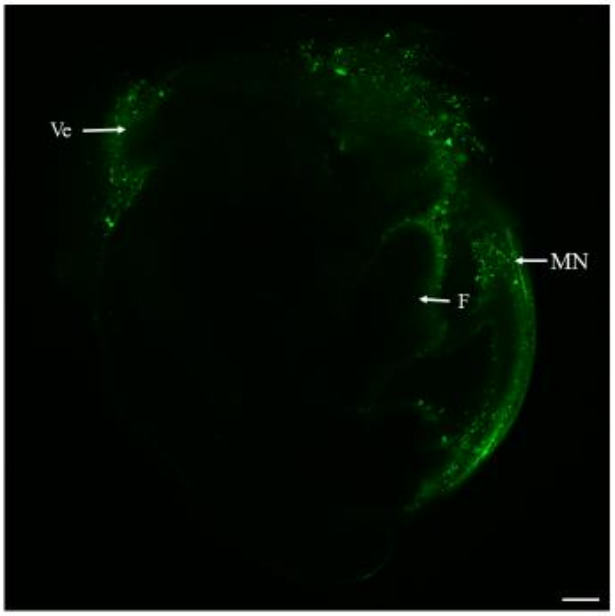

Figure 2.21.FMRFamide-like green immunoreactivity in a 21 dpf larva. Abbreviations: F- foot; MN- mantle nerve; Ve- velum. Scale bar = 20µm.

2.6 Discussion

Because of strong interest by researchers, a vast amount of literature is available as far as bivalves are concerned .A few aspects of bivalves have been a special point of focus for researchers, ranging from filter feeding, shell growth, settlement, organogenesis, gamete quality and metamorphosis (Bayne, 1971; Cardona-Costa et al., 2010; Gosling, 2003; Ockelmann, 1965; Zardus & Martel, 2002). Recently, neurogenesis has been a point of attraction for researchers, quite a few advancements have been made, which are important during bivalve larval development and settlement in order to form a successful spat (Dyachuk et al., 2012; Todt et al., 2008; Voronezhskaya et al., 1999; Voronezhskaya et al., 2008).

Unfortunately, in spite of being globally cultured, literature on shell growth, morphology, feeding consumption, organogenesis and neurogenesis of *Crassostrea gigas* is lacking (But follow,Chanley & Dinamani, 1980; Christo et al., 2010; Dinamani, 1976; Fujita, 1929; Hu et al., 1993). However, there are a few studies describing the biology, ecology and distribution of *Crassostrea gigas* around the world (Lapegue et al., 2006; Miossec et al., 2009; Pauley et al., 1988). A few workers have investigated the gamete quality of Pacific oysters in order to improve oyster spat resources (Cardona-Costa et al., 2010; Lango-Reynoso et al., 2000; Massapina et al., 1999). Seasonal changes in sexual patterns in the mature of Pacific oysters has also attracted some interest from researchers (Dinamani, 1987; Katkansky & Sparks, 1966; Ren et al., 2003). Several papers have been published based on the neurogenic studies of bivalves, especially mussels and oysters (Croll & Dickinson, 2004; Croll et al., 1997; Croll & Voronezhskaya, 1996; Flyachinskaya, 2000; Voronezhskaya et al., 1999; Voronezhskaya et al., 2008), but there is hardly any studies on this topic for *Crassostrea*

gigas. However, a study by Coon and Bonar (1986) based on the effects of epinephrine and dopamine content in *Crassostrea gigas* larvae revealed that both epinephrine (NE) and dopamine (DA) were present in appreciable quantities. Comparing these two quantities, the amount of DA was more than that of NE. The study also supported the hypothesis, that NE plays an important role in metamorphosis of larvae. There are a few other studies based on settlement cues in Pacific oyster larvae (Coon et al., 1985; Fitt et al., 1990). Coon et al. (1985) treated the larvae with selective neuroactive compounds and structural analogs to test the ability of these drugs to induce settlement and metamorphosis. The study found out that L-DOPA induced both settlement and metamorphosis, whereas epinephrine and nor-epinephrine were able to induce metamorphosis only and not the settlement. A good understanding of all the important aspects is vital for development of successful hatchery conditions, so that maximum number of healthy larvae can reach settlement and metamorphose increasing overall yield of oysters. The present study aims to improve our understanding of larval development from D-stage trochophore to settlement at about 22 days post-fertilisation with special attention directed towards survival, feeding consumption, shell length, shell morphology, organogenesis and neurogenesis. To achieve the above aim, this study used a multidisciplinary approach (Light microscopy, SEM, Confocal microscopy, immunochemistry and visual observations).

2.6.1 Survivability

In present study, the larvae were fed on a mixed diet of 1:1 *Isochryis galbana* and *Chaetoceros calcitrans,* and a linear pattern of decreasing survivability over the total rearing period of 22 days was observed, with only 2.07% of total larvae reaching settlement in the Cawthron Ultra Dense Larval Rearing System (CUDLS). These results

are similar to the findings of (Rusk, 2012), who observed that the survivability of *Perna canaliculus* larvae was just < 4% after 21 days rearing period in CUDLS. These larvae were also fed on the same diet as our present study. Another study by Wang et al. (2011) on blue mussel larvae had only 9% of controls reaching settlement. Findings by Paredes et al. (2012) were almost similar to above results as the survival rate in controls for *Perna canaliculus* was just 26% at day 18 post fertilisation. All the above studies have faced a common problem that is low survivability of controls under hatchery conditions. The potential reason for the lower survivability can be low lipid reserves in oocytes and also within feeding larvae, which are needed by the larvae when they undergo metamorphosis.

A study conducted by Soudant et al. (1999) in which the lipid content of *Crassostrea gigas* was analysed during the reproductive phase in natural as well as under artificial conditions indicates that lipid content increased and accumulated in the gonads, In the naturally conditioned animals, this accumulation was higher than in artificially conditioned animals. The above study show that oysters spent most of their energy in gametogenesis compared to somatic growth. Contrary to the above finding, Gallager et al. (1986) studied the relation between total lipid content, growth and survival on three species of bivalve larvae (*Crassostrea virginica*, *Ostrea edulis* and *Mercinaria mercinaria*). The study concluded that high survivability was always accompanied by high lipid indices, and suggested that lipid index may be used to document visually the lipid content in the individual bivalve larva as an indicator of physiological condition and potential for successful metamorphosis. The study also emphasised that the threshold lipid level in eggs is necessary for survival through the non-feeding embryonic period but environmental and genetic factors are also responsible to some extend for high mortality during the larval period. Another reason for low survivability

can be less genetic variability as it is directly related to number of parents used for spawning. as explained by Boudry et al. (2002). According to their study, it is a norm for hatchery practices to produce large number of offsprings from a limited number of parents as *Crassostrea gigas* possess high fecundity. The number of parents used for spawning are limited leading to low genetic variability. Effective population sizes can also be reduced by unbalanced contribution of each parent. The present study also followed the same norm of using limited number of parents to produce high larval population, leading to potentially low genetic variability.

Past studies indicates that dietary deficiency also limits the growth of *Crassostrea gigas* larvae to a great extent. Langdon and Waldock (1981) studied the effect of algal and artificial diets on growth and fatty acid composition of *Crassostrea gigas* spat and found great variability in the survival rates. Another study conducted on the performance of *Crassostrea gigas* larvae fed with fresh and preserved *Pavlova lutheri* concentrates in combination with fresh *Chaetceros calcitrans* revealed good growth rate and high survival rate when fed with monospecific diet of *Chaetceros calcitrans.* While explaining the broodstock conditioning of *Crassostrea gigas* Chávez-Villalba et al. (2002) described, the combination of *Isochrysis* sp. and *Chaetoceros calcitrans* is ideal for the maximum growth and survival of the larvae. Whereas Muranaka and Lannan (1984) suggested that fecundity of broodstock was 60% greater when fed an algal food supplement of the diatom *Thalassiosira pseudonana,* although the rate of gonadial development and gamete viability was not significantly different. In our present study the larvae were fed according to the broodstock conditions suggested by Chávez-Villalba et al. (2002) but low survivability indicates that feeding mixture can be more standardised.

Overall, this study indicates that there was high variability in the larval survival rate of *Crassostrea gigas* within controlled larval rearing environment and emphasise the need to develop the successful conditioning methods to increase the survival rate. Species-specific requirements for good larval survival are sometimes not well understood as the survivability depends on various biotic and abiotic factors. Hence, there is a high need to investigate these species-specific requirements to optimise the broodstock conditioning.

2.6.2 Feeding consumption

Feeding consumption of *Crassostrea gigas* larvae throughout the 22-day rearing period was analysed. At 6 dpf, larvae showed highest consumption of microalgae over the next 24 hrs. This increase in microalgal consumption can be due to high energy requirement of the larvae to undergo organogenesis and shell formation, as the growth rates are high for small larvae. Similar observations were made during the rearing of *Perna canaliculus* larvae (Rusk, 2012). The study on feeding consumption of *Perna canaliculus* larvae over the rearing period of 21 days reported that feeding consumption took a linear increase until 18 dpf before plunging down once again at day 14 (Rusk, 2012). After day 18 there was a drastic decrease in algal consumption until the end of rearing period. However, larval rearing of *Perna canaliculus*, which used CUDLS and same dietary supplements as our present study, following the same experimental design, show a steady rate in clearance rate of algal supplement (Ragg et al., 2010). Findings in the above study were quiet contrary to the present study on *Crassostrea gigas* larvae as well as *Perna canaliculus* larvae studied by (Rusk, 2012). In our study, the variability in feeding pattern may be accounted for by the different larval needs at different stages of growth. An increase in microalgal consumption just before entering metamorphosis and

settlement stage may have been due to the high energy requirement of the larvae, preparing for metamorphosis as explained by Da Costa et al. (2011) in case of Razor clam *Solen marginatus* The above study explained the incapability of bivalve larvae to feed during the transit period as the larval feeding organ (velum) is replaced by the adult feeding organ (the branchia). An important study conducted by Powell et al. (2002) on *Crassostrea gigas* larvae revealed that competency in metamorphosis is triggered by a decrease in neutral lipid and if this low lipid level is maintained during transit, it would impair successful metamorphosis. Successful completion of metamorphosis occurs if the larval neutral lipid pool is greater than the polar lipid pool, which establishes a minimum energy requirement needed to sustain metamorphosis. If this condition is not satisfied, the metamorphosis is unsuccessful and larva dies. The above reason may also account for decrease in feeding consumption, in the present study, after day 18, when the larvae started preparing for metamorphosis.

Another study conducted on *Crassostrea gigas* show variable filtration rates at different stages of growth, although experimental conditions and algal concentrations were kept constant. The feeding pattern of *Crassostrea gigas* was thought to responsible for this variability, as *Crassostrea gigas* have defined feeding period and periods of extracellular and intracellular digestion (Gerdes, 1983). These phenomenon can also account for the variable feeding consumption in the present study. A recent study on *Crassostrea gigas* larvae in semi-closed ponds revealed that the larvae showed maximum weight gain when fed on the diatom *Skeletonema costatum* (Soletchnik et al., 2001). Another study on *Crassostrea gigas* rearing revealed, there is a significant increase in survivability and growth, when the larvae were fed on combination of algae and bacteria than when the larvae were grown on algae alone (Douillet & Langdon,

1993). The algae used to feed the larvae was *Isochrysis galbana* and the medium was inoculated with strains of marine bacteria.

Hence, the present study emphasises the need to optimise dietary requirements of *Crassostrea gigas* larvae, in order to achieve maximum growth and survivability, which will eventually lead to a large number of healthy spat.

2.6.3 Shell length and morphology

Results from the present study on *Crassostrea gigas* larvae show an average shell length growth of 10.68 µm per day during rearing period of 22 days and a strong positive correlation between the shell length and larval rearing days. The increase in shell length was positively linear until day 16 and then show a slag until the end of rearing. The metamorphic transition phase, when the larvae prepares itself for settlement phase, may be responsible for this slag, as the shell stops growing during this period. A past study on *Crassostrea gigas* larvae revealed similar linear increase in shell length in controls when fed with *Isochrysis galbana* alone, but a high growth rate of larval shell was observed when a probiotic bacteria (Stain CA2) was added. Addition of this stain enhanced the larval survival by 21-22% and growth by 16-21% as compared to the controls where larvae were fed with algae alone. (Douillet & Langdon, 1994). Another study conducted recently on *Perna canaliculus* revealed similar patterns of increment in shell length during the first 14 days and then a gradual decrease onwards (Rusk, 2012). The larvae in that study were fed on the same diet as this study, but at the rate of 80-180 μL^{-1} as compared to 40 cells μL^{-1} in our present study.

The shell morphology in bivalves, which undergoes planktotrophic larval phase is relatively similar. Therefore understanding of shell morphology at species level is very important to avoid taxonomic problems. In the present study, shell morphology of *Crassostrea gigas* show similarities and dissimilarities, when compared with other bivalve species, such as clams (Mouëza et al., 2006), mussels (Rusk, 2012), and other oysters (Chanley & Dinamani, 1980; Christo et al., 2010; Dinamani, 1973, 1976; Doroudi & Southgate, 2003; Galtsoff, 1964; Hu et al., 1993; Wassnig & Southgate, 2012). The shell morphology of *Crassostrea gigas* larvae, in the present study has been documented from D-stage until the post-veliger stage, when larvae are ready to settle.

In our study, the D-stage larvae for *Crassostrea gigas* show textured appearance with punctate and comarginal radial region. The radial region extends ventrally from the punctuate region and extends towards valve opening and the hinge region. The above observation was also done by (Chanley & Dinamani, 1980), while comparing *Crassostrea gigas* with other oyster larvae from New Zealand. Similar findings were also reported in case of *Crassostrea virginica* (Carriker & Palmer, 1979). Waller (1981), while describing *Ostrea edulis* (European oyster) also reported the pitted punctuate region but the study suggested its secretion as one time event, whereas the growth of stellate radial region is achieved by increments in calcification of the shells. The pitted punctuate region as well as stellate radial region collectively constitutes PI region which is secreted by the shell gland (Waller, 1981).

The results of the present study show that as the larvae develop at 5 dpf, the umbo started developing and secreted Prodissoconch II (PII). The PI /PII boundary is clearly demarcated. This demarcation was also observed in other bivalves (Ockelmann, 1965) According to Ockelmann (1965), the PI/PII boundary demarcation is due to abrupt

changes in mode of calcification of shell calcification. The study further explained that PI is secreted by the shell gland whereas PII is secreted entirely by the mantle. However, Waller (1981) disagreed with above reasoning and proposed that the boundary represents time in the development of shell at which valves first completely enclose the body. The PII region showed distinct comarginal striate region possessing growth ridges. The study also emphasised that the PI/PII boundary also coincided with origin of postero dorsal notch and its growth track. In the Family Ostreidae, various researchers have documented presence of postero dorsal notch, as in *Saccostrea echinata*, *Ostrea circumpicta*, *Ostrea denselamellosa*, *Ostrea futamiensis*, *Ostrea edulis*, *Crassostrea virginica*, *Crassostrea ariakensis* and *Crassostrea gigas* (Tanaka, 1960), in *Crassostrea virginica* (Carriker & Palmer, 1979), in *Ostrea edulis* (Waller, 1981) and in *Saccostrea glomerulata* and *Crassostrea gigas* (Chanley & Dinamani, 1980). Chanley and Dinamani (1980), referred the postero dorsal notch in *Crassostrea gigas* as posterior dorsal sulcus, that extends from the posterior end of PI, across PII in left valve and ends in a small emargination at posterior margin of larval shell. This characteristic feature of family Ostreidae, postero dorsal notch, was also observed in *Crassostrea gigas* larvae in our present study and its demarcation was also present.

At early umbo stage larvae, the umbones were found to be round in shape, but as the larvae proceed to late umbo stage, umbones became more prominent. Chanley and Dinamani (1980) described umbo of *Crassostrea gigas* to be more skewed and knobby in appearance as compared to *Saccostrea glomerulata*. The study revealed that umbo in left valve is larger than right valve, hence creating a pronounced inequivalve condition that increases with the shell growth. All these observations agree with the present study. However, while comparing different oyster species, Chanley and Dinamani (1980) reported absence of postero dorsal notch in the case of *Ostrea lutaria* and *Ostrea*

chilensis. However, an oval hinge and clearly demarcated PI and PII regions were observed. The shells of *Ostrea lutaria* and *Ostrea chilensis* were non-umbonate and equivalve (Chanley & Dinamani, 1980). Another separate study conducted on *Ostrea edulis* revealed the unequivalve condition of shell. Clearly demarcated PI/PII boundary and presence of postero dorsal notch on the left valve of shell (Waller, 1981). Postero dorsal notch in the larvae of *Crassostrea gigas* in present study show a small gap in two valves, which is described as the absence of leading edge in case of *Ostrea edulis* (Waller, 1981). The study by Waller. (1981) describes the presence of post anal ciliary tuft, along the posterodorsal margin of shell. The study reported that beating movement of cilia deforms the shell margin. This deformity was also observed in the present study. The presence of posterodorsal notch is also documented in *Saccostrea glomerata* and a past studies on *Crassostrea gigas* (Chanley & Dinamani, 1980) Developing further, post-larva was observed to develop a distinct dissoconch layer, which is clearly demarcated from PII. The texture of dissoconch is more like an irregular flattened layer and when the larva undergoes metamorphosis, dissoconch gets cemented to substratum (Waller, 1981). The development of dissoconch layer has also been documented before in previous studies on *Perna canaliculus* (Rusk, 2012).

Overall, the shell morphology characteristics, which were observed during this study revealed great similarity to other oysters like *Ostrea edulis* (Waller, 1981), *Saccostrea glomerata* (Chanley & Dinamani, 1980) and Greenshell™ mussel (Rusk, 2012)and also dissimilarity to *Ostrea lutaria*, also known as dredge oyster and *Ostrea chilensis* (Chanley & Dinamani, 1980). Considering the shape of shell, which is usually round, members of the family Pteriidae, exhibit exceptional shell shape. The embryo acquires conical shape after entering trochophore stage. A study conducted on *Pinctada margaritifera* revealed the change in shape of shell as it enters trochophore stage from

ovoid to conical instead of circular shape. This is a characteristic phenomenon in the family Pteriidae (Doroudi & Southgate, 2003) and is totally different to our observations in Pacific oysters belonging to family Ostreidae.

However the present study lacks the study of hinge region and the teeth but they have been described in detail by Hu et al. (1993). Finally, our study on shell morphology emphasises further research on different aspects of shell morphology (microstructures and mineralogy) origin of the formation of shell, significance of postero dorsal notch would be highly beneficial.

2.6.4 Organogenesis

Light micrographic study on translucent shells of the *Crassostrea gigas* revealed useful information regarding the organogenesis from the very beginning of trochophore larvae through to settlement period. The present study revealed similar organogenetic development as in other bivalve larval species of oysters, mussels, scallops and clams. D-stage larvae show a little organogenesis, with cilia around ventral side of the larvae. The digestive system comprised of mouth, large stomach, intestine and anus. The digestive diverticulum was seen, possessing large digestive tubules, other observations include presence of a style sac at the dorsal end near hinge. Increase in size of style sac was observed with the growth of larvae. These observations are similar to D-stage larvae of *Crassostrea virginica* (Galtsoff, 1964) and *Perna canaliculus* (Rusk, 2012). A previous studies conducted on digestion process in *Mytilus edulis* revealed that extracellular digestion occurs inside the stomach and intracellular digestion occurs inside the digestive cells of diverticulum (Hawkins et al., 1983). Though the process of digestion was not studied in details in our study but, significant increase in the size of

stomach and digestive diverticulum with growth of larvae indicates presence of extracellular and intracellular digestion in case of *Crassostrea gigas*. Gosling (2003) also describes the presence of digestive cells in these digestive tubules in various bivalve larvae and mentioned that the microalgae on which bivalve larvae feed, is broken down in vacuoles and then taken up by these cells where intracellular digestion takes place.

At early umbo stage, the larvae are more or less circular in shape with a developing umbo. The style sac gets more prominent on dorsal side. The velum is more pronounced with sporadic beating of cilia, which aids in feeding and swimming. Development of alimentary canal continues well with a mouth on ventral side, leading to the esophagus, which connects mouth to digestive diverticulum and stomach. Though histological studies were not performed in our studies but this connection between the esophagus, digestive diverticulum and stomach was observed in the study of histological sections in case of *Mytilus edulis* (Bayne, 1971). The presence of algae indicates algal uptake by larvae. Contractibility of velum is regulated by velar retractor muscles, which were clear in light microscopic pictures taken during the present study. All these important aspects of development have also been reported in case of *Crassostrea virginica* (Galtsoff, 1964) and *Perna canaliculus* (Rusk, 2012). Cragg (1985) studied the pattern and function of retractor and adductor muscles in case of *Pecten maximus*. The above study revealed that these muscles become functional during early veliger larvae as was observed in our study, where velum was seen extended in some samples and contracted in some others. The proper mechanism of retraction of velum and foot has also been described in detail for *Pecten maximus* (Cragg, 1985).

As the larvae progressed to late umbo stage, organogenesis progressed further with umbones getting prominent. A large conspicuous velum with strong cilia to accommodate with increased feeding behaviour was observed, stomach and digestive diverticulum increased in size containing greater quantity of microalgae. The digestive system ending in rectum and a small anus was observed. Anterior adductor muscle, which is destined to degenerate when larvae undergoes metamorphosis, had been observed. These observations were similar to the findings of Galtsoff (1964) in case of *Crassostrea virginica* and Rusk (2012) in *Perna canaliculus*. Anterior and posterior adductor muscle have been described in detail in *Pecten maximus* (Cragg, 1985). Another important mechanism which is characteristic feature of *Crassostrea*, called pseudofeces rejection was closely observed in our present study and can be seen in the light micrographic picture at day 19. In the study of light micrograph, a big cloud of pseudofeces was seen being expelled out from the mantle surface just near to mouth. This mantle rejection mechanism enables the organism to reject particles which are not suitable for digestion. The mechanism of pseudofeces rejection has been studied in detail in *Crassostrea gigas* and *Crassostrea virginica* (Beninger'f & Veniot, 1999), and in *Crassostrea gigas* (Beninger & Cannuel, 2006), in which pseudofeces have been described as the rejection from the mantle tract, which is essential to prevent fouling and unnecessary re-treatment in suspension feeding system. The above studies on *Crassostrea gigas* and *Crassostrea virginica* describes presence of specialised ciliated cells on the mantle surface which helps in rejection of unwanted particles from mantle surface. The beating of these cilia are independent to the beating of rest of cilia present on mantle surface (Beninger'f & Veniot, 1999).

At the pediveliger stage, when larvae were preparing itself to undergo metamorphosis, organogenesis proceeded further. Larvae possessed a functional foot covered with

strong cilia, gill rudiment and an eye spot. Gill rudiment comprised of tubular channels, is located between base of foot. The style sac greatly increased in size as well as the digestive tubules. As the larvae undergo metamorphosis, velum was observed to be completely withdrawn in shell cavity and the shells were completely closed. At this stage, the organs became undistinguishable. This developmental sequence goes in accordance as observed in *Ostrea edulis* (Erdmann, 1935; Waller, 1981) and *Crassostrea virginica* (Galtsoff, 1964).

The most important characteristic features in pediveliger larvae of *Crassostrea gigas* were found to be the presence of functional foot and eye spot. The functional foot was seen lying posteriorly with in the shell but visual observations show that, for shorter periods of time it extended out of the shell but quickly seen contracting back at high speed. This retraction is caused by the movement of pedal retractor muscles as explained in case of *Pecten maximus* (Cragg, 1985) and *Crassostrea virginica* (Galtsoff, 1964). The study on *Crassostrea virginica* also explains the presence of byssal gland on base of the foot, having a small opening inside the mantle cavity. Both the foot and the gland are typical larval structures which disappear after performing their function during attachment. A pair of statocysts is also present on the base of foot, which are basically the larval sense organs (Galtsoff, 1964). Visual observations show the dark pigmented eye spot developing towards end of larval life. Appearance of an eye spot indicates the larva is nearing metamorphosis as explained by (Galtsoff, 1964) in case of *Crassostrea virginica*. Presence of eye spot was also reported in *Mytilus edulis* (Bayne, 1971) and *Perna canaliculus* (Rusk, 2012).

In commercial hatcheries, sometimes metamorphosis and settlement is induced artificially using different chemical cues. In case of Pacific oyster, *Crassostrea gigas*

larvae Coon et al. (1985) revealed that on treatment with L-3,4-Dihydroxyphenylalanine (L-DOPA), larvae undergo both settlement and metamorphosis, whereas epinephrine and norepinephrine induced metamorphosis only, without settlement. In contrast to the above finding, Croll et al. (1997) reported that catecholamines (epinephrine and norepinephrine) triggers both metamorphosis and settlement in case of *Mytilus edulis*. Effect of chemical cues on settlement have been also reported in *Pecten margaritifera* (Doroudi & Southgate, 2002). The study revealed that epinephrine or norepinephrine had no effect on settlement of the larvae, but when treated with γ-aminobutyric acid (GABA), underwent effective settlement.

Overall larval organogenesis in larval oysters depends on the number of factors, which in turns decide the rate of development of different stages. Strathmann et al. (1993), while working on *Crassostrea gigas* larvae explained the effect of nutritional gain on organogenesis. The study revealed developmental variability in velar growth in respond to food availability. The larvae provided with surplus amount of algae grew faster, eye spot developed earlier and finally they reached pediveliger stage sooner than the larvae with limited amount of algae. Surprisingly, in later case, the larvae developed wider velar lobes as compared to shell length. Cilia present on velum grew longer. Similar phenotypic plasticity was observed in case of echinoid larvae *Dendraster excentricus*, in which increase in arm length and ciliated band length was observed when the food was scarce (Hart & Strathmann, 1994). Another study conducted on *Crassostrea gigas* revealed that the digestive gland of larvae turns brick red in colour when they ingest a dinoflagellate *Prorocentrum micans*. The pigment responsible for the red colour was water soluble peridinin–chlorophyll α–protein complex (Hata & Hata, 1982) as compared to brown colour in our study where the larvae were fed on mixture of

Isochrysis galbana and *Chaetceros calcitrans* suggesting phenotypic plasticity in *Crassostrea gigas* larvae.

2.6.5 Neurogenesis

Our study attempted to study the process of neurogenesis in *Crassostrea gigas* larvae for the first time as it lacks completely in available literature for this species. However, due to lack of understanding in methodology and inadequate samples, the poor quality pictures could not explain much. The D-stage larvae exhibit very small amount of FMRF amide like radioactivity indicating little amount of neuronal development. The above observation goes in accordance with the observations in *Perna canaliculus* (Rusk, 2012) and *Mytilus trossulus* (Voronezhskaya et al., 2008). The results are comparable as the mode of larval development and settlement in *Perna canaliculus* and *Mytilus trossulus* is almost similar to *Crassostrea gigas*, characterised by free swimming, non-feeding trochophore larvae, which finally settles on the substratum following metamorphosis. Further at day 7 dpf, the mantle nerve shows more innervation of neurons and the mantle cavity also indicated high FMRF immunoradioactivity. This observation is similar to *Perna canaliculus* where (Rusk) explained mantle nerve encompassing the mantle region. At day 21 dpf, highly innervated foot was observed which indicated that foot was highly innervated by neurons, but this is contrary to the findings in *Perna canaliculus*, where no FMRF activity was observed in foot and velum (Rusk, 2012). But our studies supports the role of pedal neurons in settlement behaviour of Pacific oysters.

However, the study of central and peripheral nervous system was incomplete in our project, as no ganglia or connectives were studied, but we emphasise detailed study of

Crassostrea gigas nervous system as it is important to establish the role of neurons in certain larval behaviours such as settlement and metamorphosis.

2.6.6 Summary

In summary, this study characterise the larval development of *Crassostrea gigas* under normal hatchery rearing conditions. All the parametric data and shell morphology organogenesis and neurogenesis development exhibit overall similarities to other oyster and mussel species such as *Crassostrea virginica* (Eastern oyster), *Perna canaliculus* (Greenshell mussel), *Mytilus trossulus* (Bay mussel) and *Mytilus edulis* (Blue mussel) which leads planktotrophic life. As far as survivability is concerned, low survivability at settlement stage was similar to the past studies on *Crassostrea gigas* larvae, *Perna canaliculus* and other bivalve larvae. High feeding consumption on day 5 and 11 dpf was thought to be because of increase in organogenesis, while low feeding rates between day 19 and 22 dpf were explained by high mortality rates and process of metamorphosis as was seen in other past studies on *Crassostrea gigas* and other bivalve larvae such as *Crassostrea virginica*, *Perna canaliculus*, and *Solen marginatus.* The overall shell length observations during early stages and later during settlement period were similar to past studies on bivalve larvae. Shell morphology revealed similar results as observed in other bivalve larvae such as presence of pitted punctuate region called PI and stellate radial region called PII. Presence of characteristic postero dorsal notch is similar to past studies on *Crassostrea gigas, Saccostrea glomerulata, and Crassostrea virginica*. Organogenesis observations from D-stage through to metamorphosis show overall similarities to other bivalve larvae, particularly observation of release of pseudofeces was similar to the past studies done on *Crassostrea gigas* and *Crassostrea virginica*. However, neurogenesis studies revealed no significant results. Finally, this

study emphasise further in-depth study from embryogenesis through to post-settlement, to improve hatchery rearing conditions for *Crassostrea gigas* species.

CHAPTER 3 Cryopreservation

3.1 Abstract

Cryopreservation experiments on the D-stage larvae of the Pacific oyster (*Crassostrea gigas*) were conducted to study the impact of cryopreservation on the larval development from day 1 to 22 days post fertilisation (dpf). Two different cryoprotectant solutions and three different cooling rates were considered. Cryoprotectant solutions were made up to final concentrations of 10% ethylene glycol (EG), 1% Polyvinylpyrollidone (PVP) and either 0.2 or 0.4 M trehalose (after 1:1 dilution with larvae). Three different cooling rates of 0.5, 1, 2˚C min^{-1} between -10 and -35˚C post holding were tested in an orthogonal design with the above mentioned cryoprotectant solutions. Parametric data for survivability, shell length and feeding consumption were considered along with the larval development. Larval development was assessed through scanning electron microscopy (SEM), confocal microscopy, light microscopy and intense visual observations. Results indicate that control larvae out-performed all cryopreservation treatments for survival, feeding consumption and shell length parameters. Larvae showed normal development and 15,533 ± 613 larvae survived to day 22. However, larvae exposed to 0.4 M trehalose performed much better than those exposed to 0.2 M trehalose, regardless of different cooling rates. As 100% mortality was observed in 0.2 M trehalose treatment by day 15, while in 0.4 M trehalose treatment, 100% mortality was observed on days 21 and 22 for 0.5 and 2˚Cmin^{-1}, respectively and only 658 ± 570 larvae survived in 1˚Cmin^{-1} exposure by day 22. To assess larval morphology and organogenesis, results from scanning electron microscopy and light microscopy were compared, indicating that treatments with surviving larvae were morphologically and developmentally similar to those in the controls.

In the present study, the aim was to investigate neurogenesis, but due to lack of sufficient data, only limited results and interpretations are presented. Results show that 0.4 M trehalose may prove to be a good treatment to be tested for further application in commercial settings. This is the first multi-disciplinary study of its kind for this species, hence the present study is a stepping stone in the field of cryopreservation of *Crassostrea gigas*.

3.2 Introduction and Literature Review

3.2.1 Basics of cryopreservation

Preservation of cells and tissues, which are susceptible to damage either by bacterial growth or chemical reactivity by cooling them to sub-zero temperatures, defines cryopreservation. At this low temperature, any chemical or enzymatic activity comes to halt. This process is considered to be one of the most important components in an effective strategy to save endangered species, as cryopreservation facilitates the storage of their gametes and larvae in a gene bank, to be used later when required (Gausen, 1993). Cryoprotection plays important role in the aquaculture sector. Stocks can be easily maintained and transferred from one hatchery to another and also can be replaced by the healthy stock if ever damaged by disease or other calamities. Selective breeding can also be improved using cryopreserved gametes (Chao & Liao, 2001).

Cryobanks for various tissue cultured cells, gametes, embryos and larvae has been established in many countries and are used for various biological, clinical and animal breeding research programs (Chao & Liao, 2001). Cryopreservation techniques can prove beneficial for more advanced studies like gene transfer in animals.

In cryopreservation, scientists use very low temperature to preserve structurally intact living cells and tissues, However, the unprotected freezing is generally considered lethal due to high risk of cryoinjuries (Pegg, 2007). The process involves pre-treatment of experimental material followed by short term storage at 4˚C. The coolant generally used in the process is liquid nitrogen, as the cells must be able to survive repeated freezing

and thawing at such low temperatures to achieve successful cryopreservation method (Chao & Liao, 2001). Protection of experimental material from the damage caused by extensive cooling, is one of the major challenges for the cryobiologists.

The biological effect of freezing of intracellular water results in intracellular ice formation (IIF), which damages the cells. This damage can be avoided by adding cryoprotectants, which can suppress most cryoinjuries (Lin et al., 1999; Pegg, 2007; Renard, 1991). Cryoprotectants, reduce the amount of ice formed at any given temperature, simply by increasing total concentration of all solutes in the system, but to be biologically acceptable they must have low toxicity and be able to penetrate into the cell membranes. Many compounds have such properties, including glycerol, Dimethylsulfoxide, ethanediol, and propanediol (Pegg, 2007). However, various other factors, such as type and concentration used (as high concentration can damage the biological material), the equilibration time and the temperature during loading determines the effectiveness of cryoprotectant (Lin et al., 1999). The degree of cryoinjury also depends on the target cell permeability. If the cell is more permeable, it adjusts well with the cooling and freezing temperatures, but less permeable cells show low tolerance (Mazur, 1963).

Cryoprotectants can be classified in 2 categories depending on whether they penetrate the cell (permeating) or remain outside the cell (non-permeating). Both of them work hand in hand and give best results when used together. Permeating cryoprotectants such as Dimethylsulfoxide (DMSO) helps to lower the freezing point of the solution, minimise the osmotic shock by replacing the water inside the cell, hence reducing lethal intracellular ice formation (IIF) (Doebbler, 1966). On the other hand, non-permeating cryoprotectants, such as sugars and polymers helps to stabilize the plasma membrane

during the process of freezing and cooling (Meryman, 1971). However, determination of optimal equilibration time and cooling rate is very important as they determine the toxicity of cryoprotectant (Dong, 2005).

Gao and Critser (2000), summarised the process of cryopreservation and explained the mechanism of cryoinjury in the living human cells. The study suggested that the cells may be injured by factors, such as cold shock that have nothing to do with ice formation or CPA damage. Another reason for the cryoinjuries suggested in the study is the possibility of the conditions that allow the plasma membrane to "survive", but does not allow the "survival" of critical organelles inside cells (Gao & Critser, 2000). These organelles may range from mitochondria, ribosomes to the nucleus of cell. According to a review published by Chao and Liao (2001) most of the cryoinjuries are due to factors including pH fluctuation, cold shock, ice crystal formation, osmometric effect, and cryoprotectant toxicity, but most importantly the exposure of cells to the vast range of temperature fluctuations which normally ranges from 0 to - 40˚C.

However, the selection of specific cryoprotectants and their application remains a basic challenge for cryobiologists. As selection of optimal cryoprotectant solution is a preliminary step towards establishing cryopreservation protocols, hence in-depth study of their effects at tissue level as well as at cellular level is very important (Sansone et al., 2007).

3.2.2 Cryopreservation in bivalves

Interest in bivalves has increased tremendously in the past few decades, owing to their commercial prospective. However, their use is associated with some logistical

problems, including natural and seasonal constraints, conditioning broodstock, and low juvenile production. Cryopreservation provides an excellent opportunity to overcome these constraints, and ensures constant supply of good quality spat. In bivalves, the practice of cryopreservation is well established with special attention given to gametes, embryos and larvae.

Promising outcomes have been imparted by the researchers working on various, commercially important aquaculture species, such as the Pacific oyster *Crassostrea gigas* (Adams et al., 2004; Adams et al., 2011a; Adams et al., 2011b; Dong, 2005; Dong et al., 2005; Gwo, 1995; Lin et al., 1999; Paredes et al., 2013; Salinas-Flores et al., 2008, Tervit et al., 2005; Yankson & Moyse, 1991), *Crassostrea virginica* (Hughes, 1973; Yang et al., 2012), Greenshell™ mussels (*Perna canaliculus*) (Adams et al., 2009; Paredes et al., 2012; Rusk et al., 2013), Sea urchins (*Evechinus chloroticus*) (Adams et al., 2006; Bellas & Paredes, 2011; Paredes & Bellas, 2009), Pearl oyster *Pinctada sp* (Belinda et al., 2011; Choi & Chang, 2003; Doroudi & Southgate, 2002, 2003) and Blue mussel (*Mytilus galloprovincialis*) (Paredes et al., 2013; Wang et al., 2011).

Successful cryopreservation of eggs and embryos in sea urchin (*Paracentrotus lividus*) has been reported by Paredes and Bellas (2009). In the study, several combinations of cryoprotectants were used, but on the basis of toxicity, propylene glycol (PG) and ethylene glycol (EG) in combination with polyvinylpyrrolidone (PVP) was found to be best suited for cryopreservation of sea.urchin's eggs and embryo. The study also emphasised the use of high quality cryopreserved organisms for performing eco-toxicological bioassays for marine pollution (Paredes & Bellas, 2009). However, Adams et al. (2006), attempted the cryopreservation of another sea urchin (*Evechinus*

chloroticus) larvae with mixed results. The study concluded that ethylene glycol (EG) was less toxic to larvae than dimethyl sulphoxide (Me_2SO). However, no larvae survived freezing and thawing in EG. The above study further suggested detailed evaluation of the impact of plunging temperatures, holding times, seeding temperatures and slower cooling rates for the long term survival of the cryopreserved larvae.

In the case of American oyster, *Crassostrea virginica*, Hughes (1973), found that cryopreserved sperm were unable to enter the egg because of severe morphological damage, and only 2% of fertilisation was achieved. Cryoprotectant used in the study was 5-10% DMSO. Swollen and vacuolated head in cryopreserved sperm were reported in the above study. However, successful cryopreservation of sperm and larvae in *Crassostrea virginica* was achieved using 10% propylene glycol as a cryoprotectant (Paniagua-Chavez & Tiersch, 2001). Furthermore, more refined method for cryopreserving *Crassostrea virginica* sperm was published by Yang et al. (2012) claiming to produce thousands to straws per day with homogenous and reliable quality of sperm using dimethyl sulfoxide as a cryoprotectant.

Attempts to cryopreserve the gametes of *Perna canaliculus* (green-lipped mussel) have also yielded mixed results. Adams et al. (2009), suggested that the sub-lethal chilling injury was responsible for less than 1% survivability of D-stage larvae and not the intracellular ice formation (IIF). The larvae in this study were obtained using cryopreserved oocytes using ethylene glycol and trehalose. Another study conducted by Smith et al. (2012), an attempt to cryopreserve *Perna canaliculus* sperm was successful to achieve fertilisation rate equivalent to the fresh sperm but with an average requirement of 363 x more cryopreserved sperm to fertilise the same number of eggs approximately. The above results indicated that viability of fresh sperm was much more

than the cryopreserved ones. Though the above method is currently being used in selective breeding of mussels but further refinement was advised (Smith et al., 2012).

Mixed results have been reported when it comes to cryopreservation of Greenshell™ mussel (*Perna canaliculus*) larvae. Paredes et al. (2012) used different combinations of EG and trehalose. The study concluded that the best combination for cryopreservation of *Perna canaliculus* larvae was 10% EG and 0.4 M trehalose. In that study, 40-60% of trochophore larvae were able to develop to D-stage larvae when compared to controls. However, only 2.8% of cryopreserved trochophore developed into competent pediveligers as compared to 26% of the controls. Paredes et al. (2012) also emphasised need for in-depth study of impact of cryopreservation on various aspects of cryopreserved larvae such as shell morphology and organogenesis, to further optimise cryopreservation protocol. In a different study, Rusk et al. (2013), working on *Perna canaliculus*, concluded that the larvae cryopreserved at D-stage gave better results than those cryopreserved at trochophore stage. The cryoprotectant used in the study were 10% EG and 0.4 M trehalose. Rusk et al. (2013) studied the impact of cryopreservation on *Perna canaliculus* larvae, on shell length, feeding, shell morphology organogenesis and neurogenesis, from the beginning for D-stage to the settlement point as suggested by Paredes et al. (2012), revealing some important abnormalities which can be useful for cryobiologists.

Successful cryopreservation of blue mussel, *Mytilus galloprovincialis* larvae at D-stage was achieved by Wang et al. (2011), using dimethyl sulphoxide (DMSO), ethylene glycol (EG), propylene glycol (PG) and their combinations with trehalose. In that study, the most effective CPA was found to be 5% DMSO, resulted in 55.3 ± 7.8% post-thaw survival rate, which was highest among the combinations used. A preliminary study was

conducted on cryopreservation of sperm of the blue mussel, *Mytilus galloprovincialis*. The results indicated the potential cryopreservation in future using 7% ethylene glycol and also suggested that the sperm motility should not be considered as the only criteria for assessing good psychological status of thawed sperm, as membrane surface chemistry is equally important.

Liu and Li (2008) in their study on *Saccostrea glomerulata* emphasised the importance of larval age undergoing cryopreservation for the best results, with maximum survival rates achieved in 24hrs old larvae. In a separate study by Horváth et al. (2012), on European flat oyster, the authors found that none of larvae survived 24hrs post-thawing. The reason presented accounting for 100% mortality was the inaccuracy of freezing protocol. The cryoprotectants used were methanol and dimethyl sulphoxide (DMSO). The study emphasised the importance of standardized cooling conditions for every species.

Choi and Chang (2003), while working on larvae of the pearl oyster, *Pinctada fucata martensii*, emphasised on importance of optimal cooling rate during cryopreservation to avoid intracellular ice formation. The above study revealed that best larval stage to undergo cryopreservation is late D-stage larvae at the cooling rate of 1˚C using 0.2 M glucose or sucrose for best survival rate (Choi & Chang, 2003). In a separate preliminary study on the black-lip pearl oyster, *Pinctada margaritifera*, combination of 1 M trehalose and 5% DMSO were found to be best suited for cryopreservation of sperm. However, the total motility of the cryopreserved sperm was found to be 89% as compared to normal non-cryopreserved sperm. Further investigation was suggested in order to increase the motility of cryopreserved sperm.

Overall, for successful cryopreservation, the importance of use of optimum cryoprotectant solutions (CPA) along with optimum cooling rates, seeding temperatures and equilibration time have been highlighted for longer survivability of cryopreserved larvae (Chao & Liao, 2001; Horváth et al., 2012; Paniagua-Chavez & Tiersch, 2001; Paredes & Bellas, 2009). The stage of development (D-stage or trochophore) at which the larvae are cryopreserved matters a lot as later stages of development such as D-stage are more resilent than trochophore stage to cryopreservation and high survival rates are obtained (Choi & Chang, 2003). Furthermore, the need to analyse the effect of cryopreservation on the larval quality not just post-thawing but over a longer period of development has been advised (Paredes et al., 2012; Wang et al., 2011).

3.2.3 Cryopreservation on Crassostrea gigas

Fortunately, a vast amount of literature is readily available on cryopreservation of gametes and embryos of *Crassostrea gigas*, as these aspects have been extensively investigated (Adams et al., 2004; Adams et al., 2011a; Adams et al., 2011b; Chao et al., 1997; Dong et al., 2005; Gwo, 1995; Lannan, 1971; Lin et al., 1999; Paredes et al., 2013; Renard, 1991;Salinas-Flores et al., 2008; Salinas-Flores et al., 2008; Tervit et al., 2005). An attempt to produce larvae from cryopreserved eggs has also been established successfully (Tervit et al., 2005). However, there is not much advancement being made in field of cryopreserving *Crassostrea gigas* larvae, with only a limited literature available, although later stages have been suggested to be more resistant to cryopreservation stress (Gwo, 1995; Nascimento et al., 2005; Suquet et al., 2012).

Efforts to cryopreserve the semen of *Crassostrea gigas* started long back in 1971 using 20% DMSO with fertility rates of only 0-10% (Lannan, 1971), as it provides genome

stability and permits guaranteed supply of sperm throughout. Later several other researchers investigated this field with mixed results. Finally cryopreservation methods for *Crassostrea gigas* sperm, have been successfully developed and are currently been used in selective breeding of this species (Adams et al., 2004; Adams et al., 2011a; Dong et al., 2005; Smith et al., 2001). Adams et al. (2004), concluded in their study, that trehalose alone was an effective cryopreservant but the most effective cryoprotectant for *Crassostrea gigas* sperm was the combination of 0.45 M trehalose with 2.5-15% DMSO.to produce commercial spat. In a separate study conducted by Dong et al. (2005), it was revealed that 100% production of triploid offsprings were obtained when cryopreserved sperms from tetraploid oysters were used to fertilise eggs from diploid females. The highest fertilization obtained for thawed sperm was 96% for sperm from diploid oysters and 28% for sperm from tetraploid oysters. Dong et al. (2005) specified the importance of triploidy in aquaculture for improved meat quality and growth because of reduced gamete output (functional reproductive sterility).

Not only the sperm, but the oocytes of *Crassostrea gigas* have been successfully cryopreserved. This research was the outcome of work by Tervit et al. (2005). In their experiment, four different cryoprotectants were tested. From these, EG and DMSO were found to be most effective with fertilisation rates of 51.0 ± 8.0 and 45.1 ± 8.3%, respectively, while propylene glycol and methanol were found to be least effective resulting in zero fertilisation. The above study also illustrates Milli-Q water as an effective base medium rather than sea water In another study, the importance of cell's osmotic tolerance and membrane permeability was highlighted in *Crassostrea gigas* oocytes to develop successful cryopreservation technique as well as importance of cryobiological characteristics of given cell type was demonstrated (Salinas-Flores et al., 2008). Embryos of *Crassostrea gigas* have also been reported to survive following

cryopreservation (Chao et al., 1997; Gwo, 1995; Renard, 1991) but, high mortality rates post-thawing have been observed revealing cryopreservation injuries (Lin et al., 1999; Renard, 1991). Renard (1991) also indicated the cooling tolerance of the Pacific oyster embryo at 2-4 cell stage varies according to embryo quality used during experiments. During the effort to cryopreserve late embryos and early larvae of the Pacific oyster and the hard clam, Chao et al. (1997) ensured the vitrification freezing method has better potential than the normal freezing method as it provides ice-free cooling.

To date, there are few reported studies on cryopreservation of Pacific oyster larvae at the D-stage, although later stages have been suggested to be more resistant to cryopreservation stress (Gwo, 1995; Nascimento et al., 2005; Suquet et al., 2012). D-stage larvae also are more resilient to handling stress than earlier stages that do not possess a shell, such as trochophores (Adams, personal observations). There are number of abnormalities and slow development rates reported, while trying to cryopreserve Pacific oyster larvae (Suquet et al., 2012; Usuki et al., 2002). In today's world, where eco-systems are changing day by day because of pollution, the cryopreserved larvae of Pacific oyster are being used to perform eco-toxicological bioassays. McFadzen (1992) used cryopreserved larvae of Pacific oyster and Manila clam to assess the biological effects on these bivalve larvae along a known contaminant gradient in the North sea, revealing high sensitivity to the on-shore pollution.

Paredes et al. (2013), obtained high yield of D-stage larvae (60% of controls) from cryopreserved trochophores, but the thawed larvae were approximately 10% smaller than those of controls. The cryoprotectants used were EG, trehalose and PVP. The study concluded that further research was needed in order to obtain a successful cryopreservation method for *Crassostrea gigas* larvae. In addition, the importance of

evaluating cryopreservation success, not just post-thawing, but over longer time periods, for example by measuring survivability, shell growth, organogenesis and successful transition to settlement, has been recognised.

3.2.4 Significance of cryopreserving Crassostrea gigas larvae

Crassostrea gigas is one of the most important aquaculture species, not only in New Zealand, where it is worth NZ$ 25 million per annum but, worldwide. The recent outbreaks of the Ostreid herpes virus (OsHV-1) in March 2010, which was responsible for mass juvenile mortality events in New Zealand broke the backbone of this country's aquaculture (as discussed earlier in the chapter) industry. Hence, cryopreservation is an important alternative source of larvae, if or when the mass mortality occurs either due to disease outbreaks or climate change. Successful cryopreservation of *Crassostrea gigas* larvae will decrease the dependency of farmers on wild-caught spat (juveniles), and also will provide an alternate supply of viable larvae on demand if the broodstock is not well conditioned due to natural or unnatural reasons. Higher rate of viable larvae may lead to reduction in broodstock conditioning cost. Another important scientific advantage of using cryopreservation is that, it offers the opportunity to select the breeding lines. More efficient and disease resistant breeding lines can result in favourable genetic stock, which requires less maintenance and are effective and reliable sources of good quality spat year after year.

Most recently, cryopreserved larvae of *Crassostrea gigas* have been used in performing eco-toxicological bioassay to analyse marine water pollution. Hence cryopreserved larvae ensures availability of high quality material to analyse ecosystem damage which is usually irreversible, all the year around.

3.3 Aim & Objectives

The aim of this chapter is to describe the effects of cryopreservation on the larval development of *Crassostrea gigas* treated with two different cryoprotectant solutions, from D-stage larvae through to post-settlement.

The objectives are:

- To describe shell morphology of cryopreserved larvae using scanning electron microscopy and light microscopy.
- To describe survivability and feeding patterns of cryopreserved larvae over the total rearing period of 22 days.
- To describe organogenesis processes in cryopreserved larvae using light microscopy.
- To describe neurogenesis in cryopreserved larvae using immunochemistry.

3.4 Material and Methods

3.4.1 Experimental reagents

For cryopreserved treatments, 3 cryoprotectants, trehalose, ethylene glycol (EG) and polyvinylpyrrolidone (PVP-40) were obtained from Sigma-Aldrich chemicals. Bovine serum albumin (BSA; Albumax I Lipid Rich BSA) was obtained from GIBCO Invitrogen Corporation, Auckland, New Zealand.

For confocal microscopy, the same reagents were used as described in Chapter 2.

3.4.2 Gamete collection, fertilization and incubation

Spawning of the mature broodstock, gamete collection and incubation is described in detail in Chapter 2 (2.4.1 and 2.4.2 respectively), Fig 2.4.

3.4.3 Cryopreservation

Cryoprotectant solutions (CPAs) were prepared using a combination of trehalose, polyvinylpyrrolidone [PVP-40], and ethylene glycol [EG]. Two CPAs were made up at twice the desired final concentration using Milli-Q water, such that the final concentrations (following 1:1 dilution with larvae) were 10% EG + 1% PVP-40 plus either 0.2 M or 0.4 M trehalose.

Concentrated larvae (1.6 – 2.1 x 10^6 mL^{-1}) were divided into 1mL aliquots in glass test tubes and diluted 1:1 with CPA at room temperature (~22°C) for a 20 min equilibration period. During equilibration, diluted larvae were aspirated into 0.25 mL straws (Instruments de Medicine Veterinaire [IMV], L'Aigle, France) and plugged by dipping the open end in sealing powder and Milli-Q water. Straws were then wiped dry with a tissue and placed into controlled rate freezers which were programmed to hold at 0ºC for 5 min, then cool at 1ºC min^{-1} to -10ºC, hold for 5 min, then cool at 0.5, 1 or 2ºC min^{-1} to -35ºC, before plunging them into liquid nitrogen (Fig.3 1).

Samples were left in liquid nitrogen for approximately 1 h before being thawed. Straws were thawed by immersing in a 28°C water bath until the ice had melted. The contents of four straws were added into 2mL of FSW containing 0.1% bovine serum albumin and left for 15 min. After this time, 500,000 larvae per treatment were transferred to the Cawthron Ultra Density Larval Rearing System , (CUDLS, King et al., 2005; Ragg et al., 2010) by taking the appropriate volume of diluted larvae (1.6 – 1.9 mL) and adding them to a rearing tank half filled with filtered seawater. A sabre shaped screen with 40 µm mesh and two glass droppers, one delivering air and the other delivering seawater and micro-algae were then added to each tank. For each cryopreservation treatment, there were three replicate CUDLS tanks (one for each pool) with each CUDLS tank stocked at a starting density of 500 000 larvae.

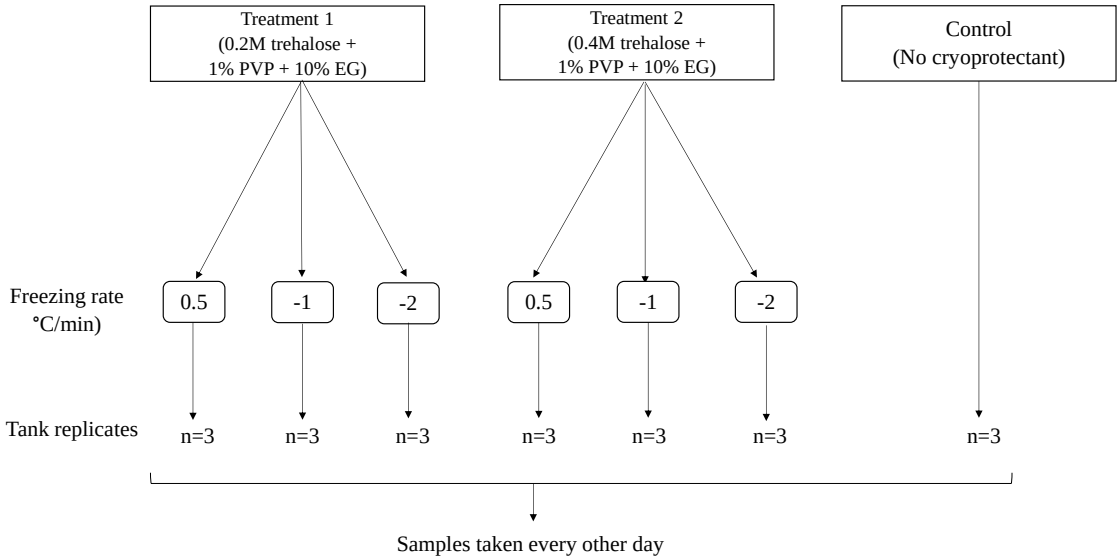

Figure 3.1.Diagram of experimental design, including treatments with two cryoprotectant solutions (10% ethylene glycol + 1% polyvinylpyrrolidone (PVP- 40) + either 0.2 M or 0.4 M trehalose) and three freezing rates (0.5, 1 and 2˚C) and controls (no cryopreservation).

3.4.4 Larval rearing and sampling

A total of 21 CUDLS were prepared, with 3 replicates for each treatment and controls at a density of 500,000 larvae per CUDLS (Fig 3.1). Out of these 3 replicates of controls were used to explain the morphology, shell growth, feeding, organogenesis and neurogenesis and the rest of 18 CUDLS were used to explain the overall effect of cryoprotectants on the Pacific oyster larvae. Samples from each CUDLS were fixed daily or alternate days for treatments until post-settlement, as described in Chapter 2-2.4. Larval rearing from day 1 until post-settlement was similar to controls. The same methodology was followed to analyse the survivability, feeding consumption and shell length as in Chapter 2-2.4.

For light microscopy, scanning electron microscopy and confocal microscopy, the same reagents and instruments were used as controls. Results from Chapter 2 were used to

compare the shell morphology (SEM and Light microscopy), organogenesis (Light microscopy) and neurogenesis (confocal microscopy) with the treatments.

3.4.5 Statistical analyses

SPSS Statistical 6 software was used to analyse all the data. For survivability and feeding consumptions, separate repeated measures ANOVA was used to identify statistical differences among control and cryoprotectants/cooling rates over a rearing period of 22 days. For the shell length data, there were insufficient samples left towards the end of experiment to measure. Hence, a separate repeated measures ANOVA was conducted to detect differences only among groups with surviving larvae (controls and 0.4 M trehalose, 1ºC min^{-1} and 0.4 M trehalose, 2ºC min^{-1} treatments).

3.5 Results

3.5.1 Survivability analyses

Survivability between controls and all other treatments over the 22-day rearing period varied significantly. Furthermore, Tukey tests following a repeated measures ANOVA detected no significant differences among cryoprotectant treatments. Generally, among all the CUDLS, controls as well as treatments, survivability % decreased sharply over the first 5 days of total 22 days larval rearing period (Fig: 3.2). However, as the experiment reached day 11, less than 5% survivability was observed in all the cryoprotectant treatments, whereas controls had more than 25% survivability. All the treatments with 0.2 M trehalose proved fatal as 100% mortality was observed by day 15. For 0.4 M trehalose treatments, 100% mortality for 0.5˚C and 2˚C exposures were observed on day 21and 22 respectively, whereas for 1˚C, only 658 ± 570 larvae survived by day 22 as compared to survival of 15,533 ± 613 larvae in controls. On day 19, all the larvae were accidently exposed to higher UV dose than normal as mentioned in Chapter 2, this overexposure may be accounted for high mortality rates between day 19 and 22. A significant interaction and pairwise comparisons between days reflect the high variability encountered among replicates and treatments.

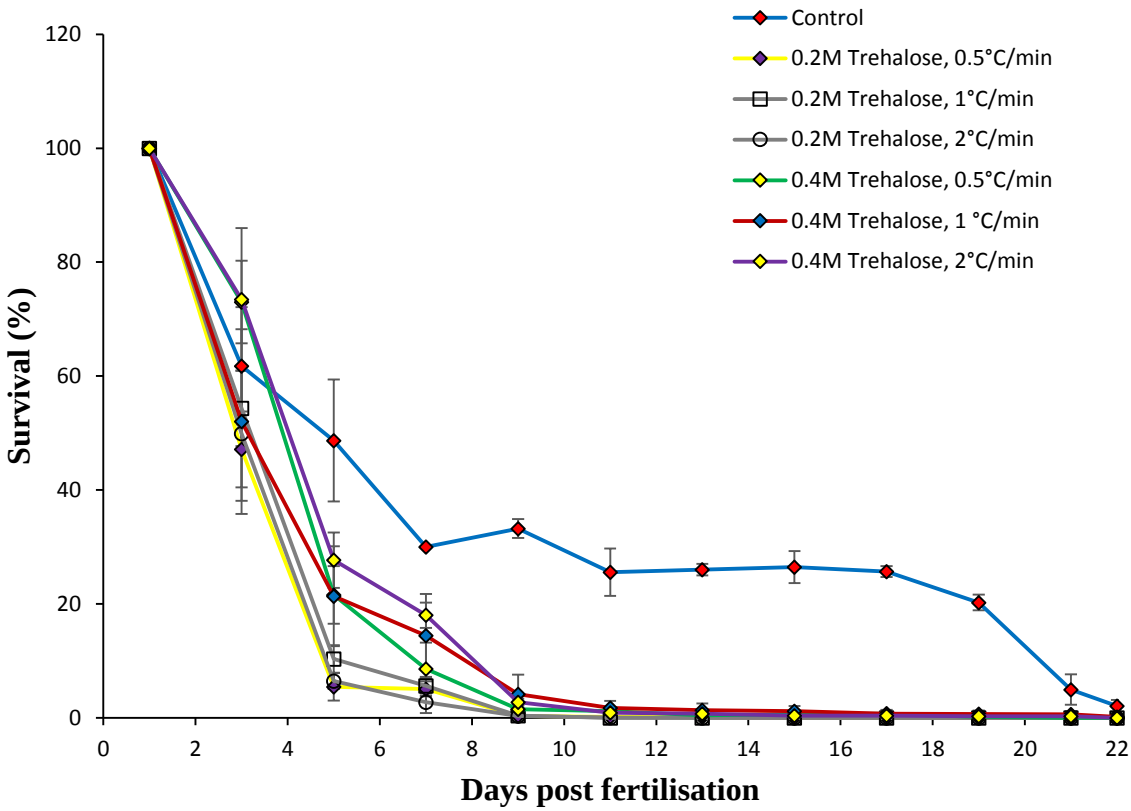

Figure 3.2.Percent survival (± SE) of oyster larvae during the 22 days rearing period within control and treatments including two cryoprotectant concentrations (0.2 M trehalose + 1% PVP + 20% EG and 0.4 M trehalose + 1% PVP + 20% EG) and three freezing rates (-0.5,-1, and -2˚C).

3.5.2 Shell length analyses

Shell length (µm) measurements among controls and all the treatments varied significantly over the 22 day rearing period. Shell length followed a gradual increase along an S-shaped curve with greater separation among controls and treatments in the later part of rearing period (Fig 3.3). Throughout the experiment, larvae in control tanks had greater shell lengths with initial shell length of 79.0 ± 2.3 µm on day 1 and a final shell length of 313.8 ± 14.2 µm on day 21. While in treatments, larvae show immediate shrinkage after adding CPAs with average shell length of 77.1 ± 1.44 µm in 0.2M trehalose treatment and 77.5 ± 0.1 µm in 0.4 M trehalose treatment (Fig 3.3). Due to insufficient larval samples toward the end of the experiment (due to high mortality), statistical analyses were performed only on available data among control, 0.4 M trehalose, $1^{\circ}Cmin^{-1}$, and 0.4 M trehalose, $2^{\circ}Cmin^{-1}$ treatments. These analyses resulted in significant differences between the control and each of the treatments, but no significant differences between treatments, indicating that controls were slightly larger than those in the other two treatments.

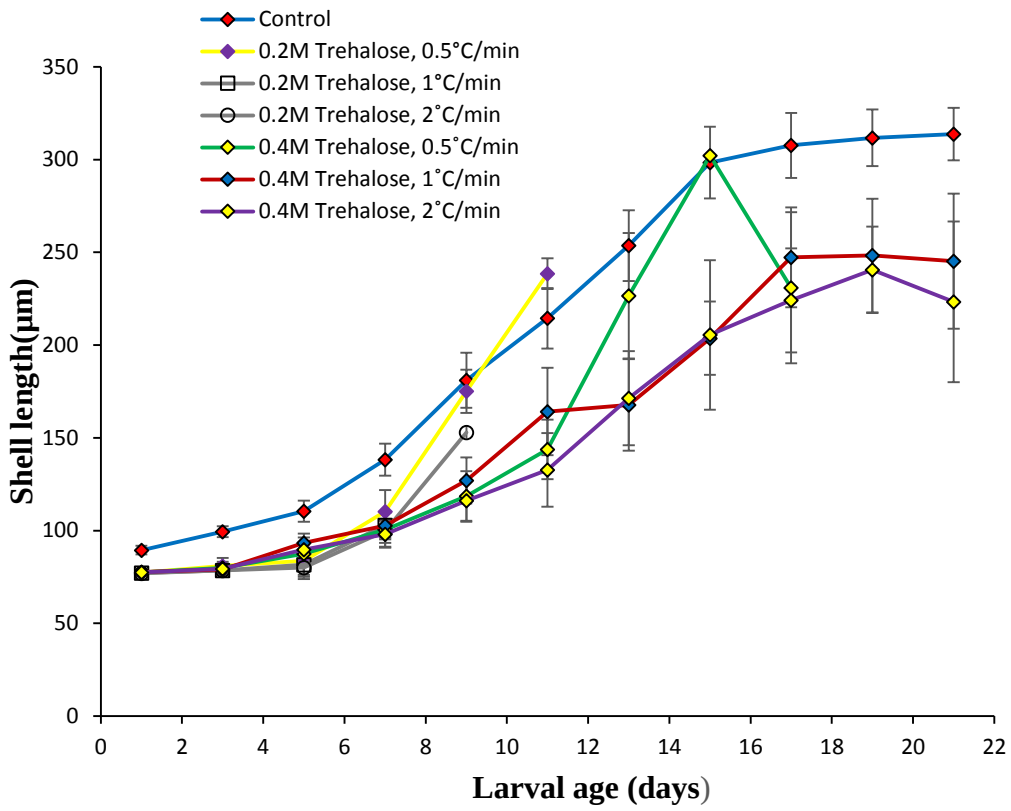

Figure 3.3.Average shell length (± SE) of larvae over 22 days within control and treatments including two cryoprotectant concentrations (0.2 M trehalose + 1% PVP + 20% EG and 0.4 M trehalose + 1% PVP + 20% EG) and three freezing rates (-0.5,-1, and -2°C).

3.5.3 Feeding consumption analyses

Feeding consumption followed a similar trend among controls and treatment tanks for the first 7 days, with a sharp increase on day 5 and then a subsequent decrease on day 7 (Fig 3.4). Following day 7, until the end of rearing period, control tanks consistently had higher feeding consumption values than the other treatments, even though there was high variability. A repeated measures ANOVA and Tukeys test detected significant differences between control and all other cryoprotectant treatments, except for the controls and 0.4 M trehalose, 2˚Cmin^{-1} treatment. Among treatments, peak microalgal consumption was recorded on day 5 with 49.5 ± 5.9% consumption in the 0.4 M trehalose, -0.5˚Cmin^{-1} treatment (Fig 3.4). Two subsequent consumption peaks were observed on day 11 and 17, especially for control tanks which had 39.5 ± 7.6mVday^{-1} and 40.5 ± 5.2mV day^{-1}, respectively. By day 19, all the controls and treatments had less than 10mV day^{-1} consumption. The high variability in feeding consumption can be associated with high mortality periods, especially on days 9 and 13.

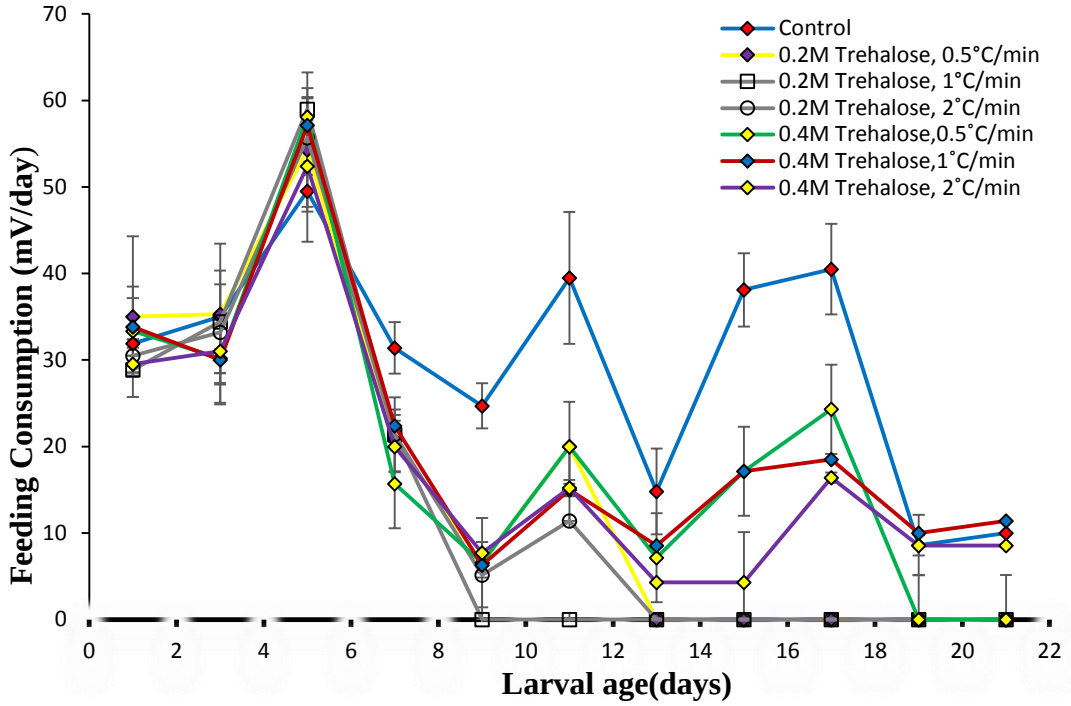

Figure 3.4.Mean percent feeding consumption (± SE) of larvae over 21 days within control and treatments, including two cryoprotectant solutions (10% ethylene glycol + 1% polyvinylpyrrolidone plus either 0.2 M or 0.4 M trehalose [final concentration]) and three freezing rates (0.5, 1, 2˚Cmin^{-1}).

3.5.4 Shell Morphology

Based on SEM photographs, control, 0.2 M trehalose and 0.4 M trehalose treatments show various morphological differences among themselves (Table-3.1), but as far as exposure to different cooling rates are concerned, no obvious differences were observed among the larvae. Thus the SEM plate reflects only three larval groups i.e. control, 0.2 M trehalose and 0.4 M trehalose.

D-stage larvae (1-day post fertilisation [dpf]) obtained prior to freezing and transferred to the CUDLS system (controls) had an average shell length of 79.0 ± 2.3µm (Fig 3.5 - 1A) as compared to 0.2 M and 0.4 M trehalose, which show slight shrinkage, with an average shell length of 77.1 ± 1.4 µm (Fig 3.5 -2A) and 77.5 ± 0.1 µm (Fig 3.5 -3A). Controls had a punctuated pitted region, showing a prodissoconch I (PI) layer with a satellite radial region extending ventrally towards the edge of the mantle (Fig 3.5 - 1A). Both cryopreserved larval groups had punctuated pitted regions showing a prodissoconch I (PI) layer with a stellate radial region extending ventrally towards the edge of the mantle but these larvae also showed some abnormalities at the shell margin (Fig 3.5 - 2A, 3A). SEM image of day 1 larvae in 0.4 M trehalose showing abnormalities at the margin can be seen, (Fig 3.6). Another SEM image of day 1 larva in 0.2 M trehalose showing distorted shape of the mantle at the edge had been observed. The shell is bit elongated to one side. (Fig 3.7). At day 5, control larvae develop PII layer with comarginal growth annulations extending from the transition zone of the PI and PII (Fig 3.5 - 1B), whereas larvae in both the cryoprotectant solutions failed to form a PII layer and had shell smaller than controls (Fig 3.5 - 2B, 3B). Controls, 0.2 M and 0.4 M trehalose larvae showed an average shell length of 110.4 ± 5.6 µm, 81.8 ± 7.7 µm

and 90.2 ± 6.5 µm, respectively. Morphologically, these larvae assumed oval appearance which is quite different to those in controls. Another SEM image of day 5 larva in 0.2 M trehalose show abnormalities on the margin as well as on the surface of the shell, this larval abnormalities are suggested to be the result of cryoinjuries and indicates that the larva is destined to die in near future (Fig 3.8).

Further progression of control larvae to 9 dpf showed the development of umbo, suggesting the onset of an early umbo stage at an average shell length of 181.1 ± 14.9 µm (Fig 3.5 - 1C). In contrast, larvae exposed to both cryoprotectants developed to the PII stage with an average shell length of 118.6 ± 13.3 µm and 152.9 ± 12.1 µm (Fig 3.5 - 2C, 3C) respectively. Few SEM images of day 9 larvae in 0.2M (Fig 3.9) and 0.4 M (Fig 3.10) has been attached showing abnormalities on the margins and the shell surface. These larvae are presumed destined to death in near future because of these abnormalities. However, low survivability after day 11 for larvae exposed to 0.2 M trehalose resulted in insufficient representative samples for shell morphology characterisation. Control larvae at 15 dpf had a well-rounded umbo shell with a further secretion of the PII layer, and an average shell length of 298.4 ± 19.4 µm (Fig 3.5 - 1D). A few larvae exposed to 0.4 M trehalose managed to reach umbo stage with an average shell length of 229.1 ± 16.3 µm (Fig 3.5 - 3D). At day 17, larvae in controls and those exposed to 0.4 M trehalose grew further in shell length and reached an average of 307.7 ± 17.6 µm (Fig 3.5 - 1E) and 231.6 ± 26.9 µm (Fig 3.5 - 3E), respectively. Though both the larvae in controls and in 0.4 M trehalose show development of postero dorsal notch, but morphologically cryoprotected larvae showed oval appearance, which is very much different to morphological shape of the controls. At day 21, control larvae reached the shell length of 313.8 ± 14.2 µm, had retracted the velum and developed a gill rudiment, which are characteristic features of progression onto a spat stage (Fig 3.5 - 1F). Few

larvae exposed to 0.4 M trehalose survived to day 22, with an average shell length of 245.3 ± 36.6 µm (Fig 3.5 - 3F). The number of cryopreserved larvae in day 22 was too low to make significant observations.

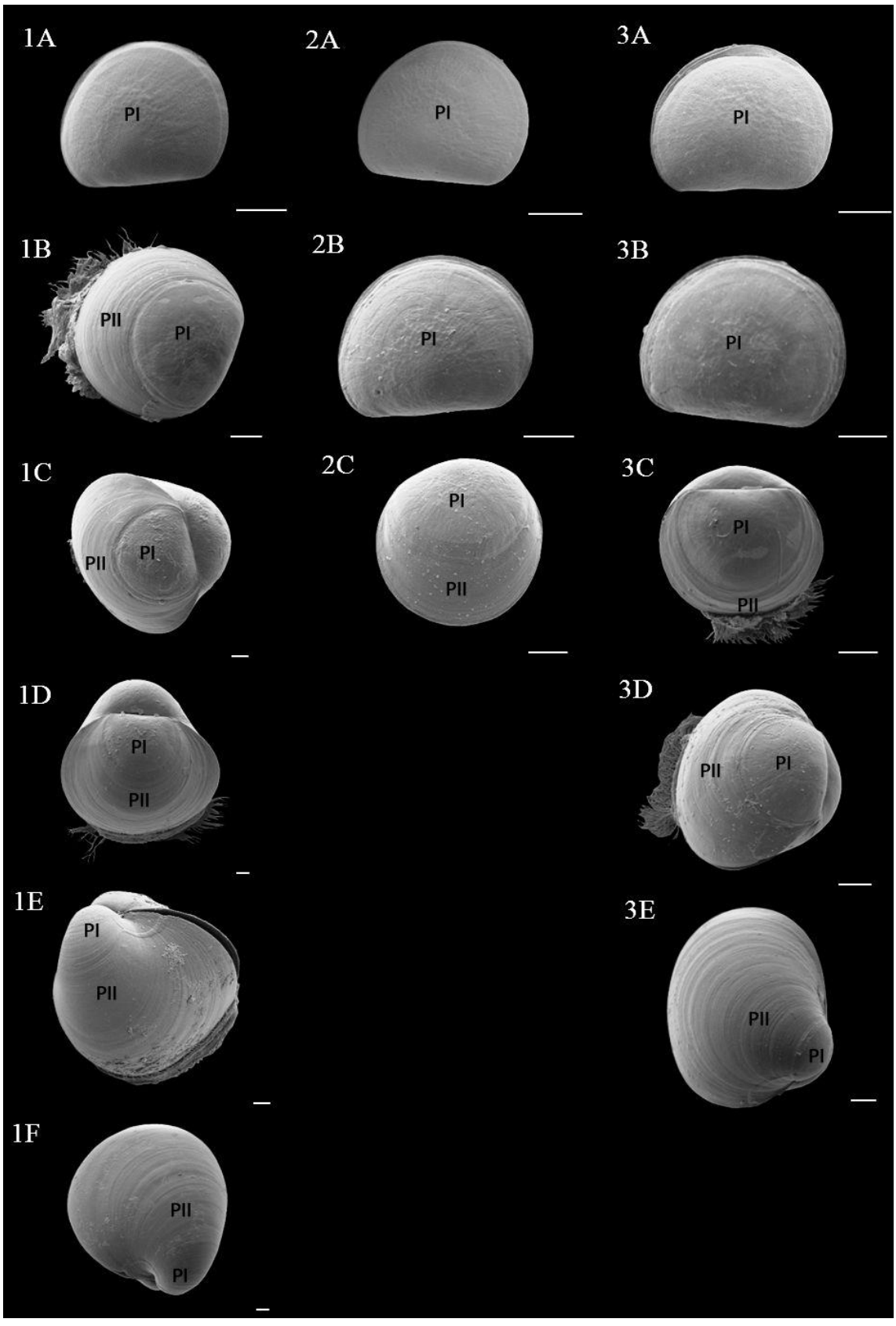

Figure 3.5.SEM images of *Crassostrea gigas* larvae over the rearing period within controls (1A- 1F) and larvae exposed to 0.2 M trehalose (2A – 2C) and 0.4 M trehalose (3A – 3F). A, B, C, D, E and F represent

larvae that were 1, 5, 9, 15, 17, and 21 days old, respectively. Abbreviations: PI- Prodissoconch I; PII- Prodissoconch II. Scale bars = 20 µm.

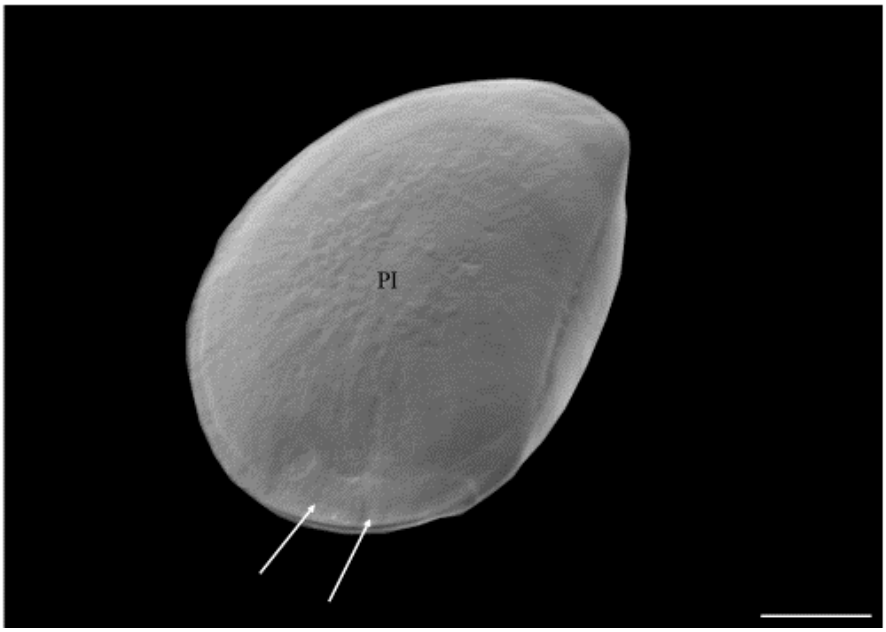

Figure 3.6.SEM image of 1 dpf larva in 0.4 M trehalose showing abnormalities at the margins. Abbreviations: PI- Prodissoconch I. White unlabelled arrows shows abnormality. Scale bar = 20 µm.

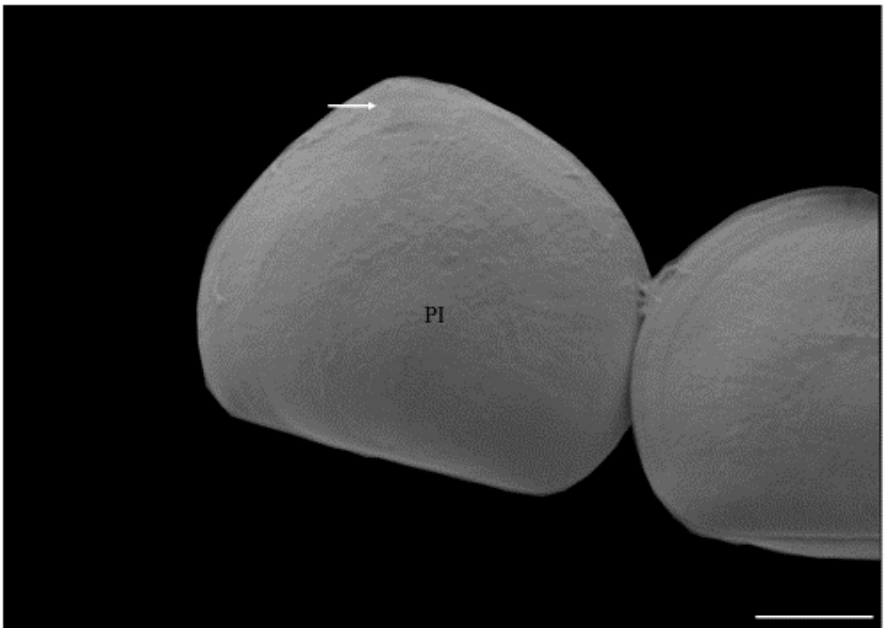

Figure 3.7.SEM image of 1 dpf larva in 0.2 M trehalose showing abnormalities at the margin. Abbreviations: PI- Prodissoconch I. White unlabelled arrows shows abnormality. Scale bar = 20 µm.

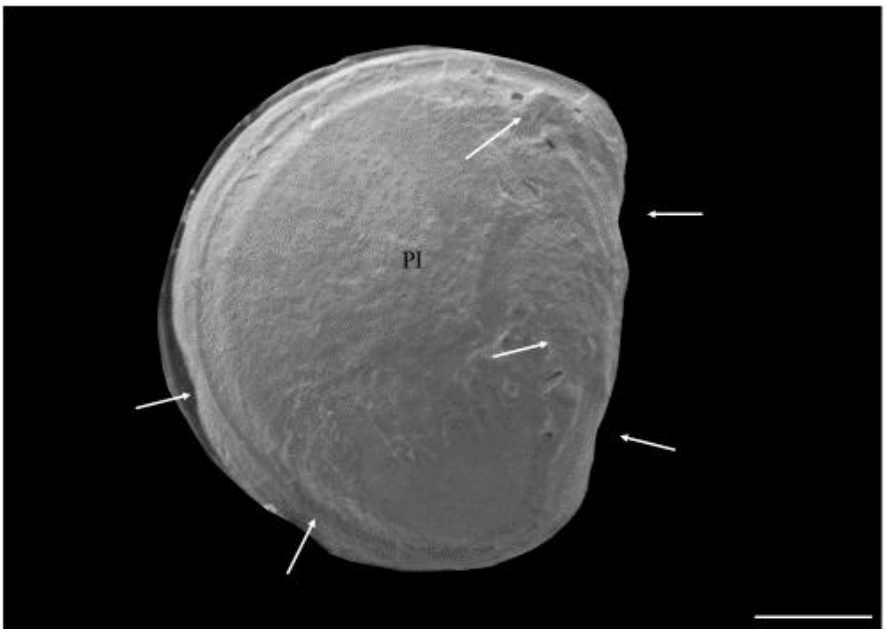

Figure 3.8.SEM image of 5 dpf larva in 0.2 M trehalose showing abnormalities on the shell margin as well as on the surface. Abbreviations: PI- Prodissoconch I. White unlabelled arrows shows abnormality. Scale bar = 20 µm.

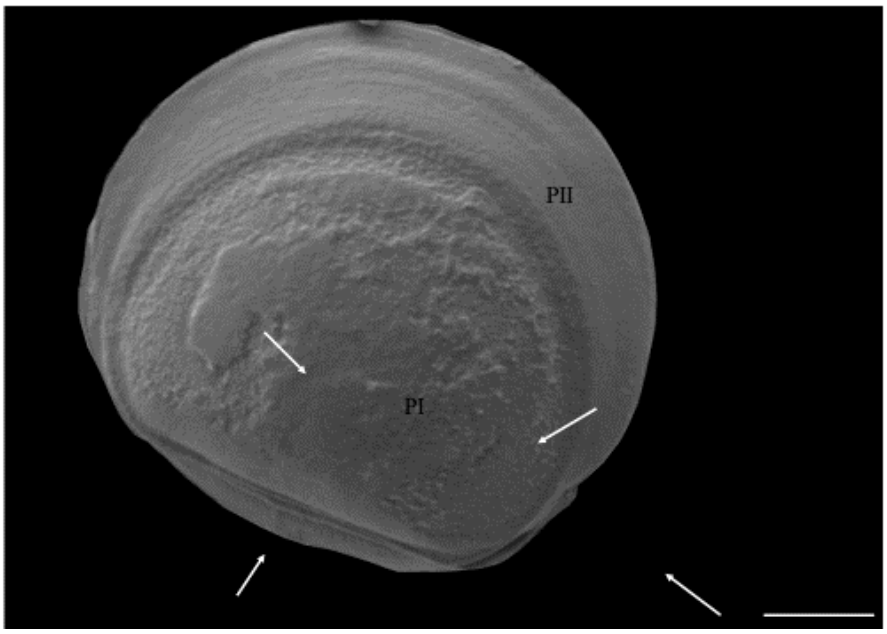

Figure 3.9.SEM image of 9 dpf larva in 0.2 M trehalose showing abnormalities at the margin as well as on the surface of the mantle. Abbreviations: PI- Prodissoconch I; PII- Prodissoconch II. White unlabelled arrows shows abnormality. Scale bar = 20 µm.

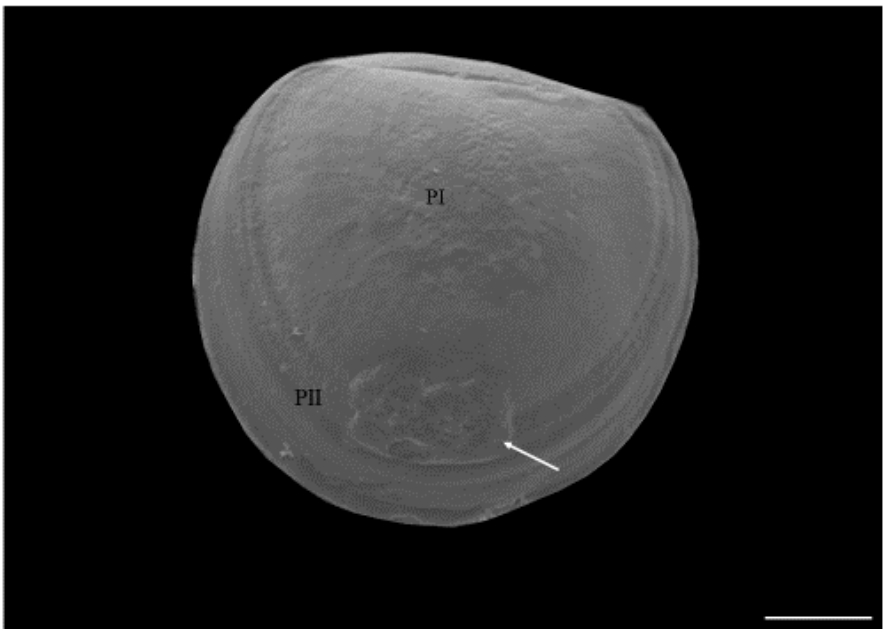

Figure 3.10.SEM image of 9 dpf larva in 0.4 M trehalose showing abnormality on the surface of the shell. Abbreviations: PI- Prodissoconch I; PII- Prodissoconch II. White unlabelled arrows shows abnormality. Scale bar = 20 µm.

Table 3.1.Different larval stages of *Crassostrea gigas* in control and cryopreserved in days (d). (*) - Nil survivability of larvae.

Time post fertilisation	Control	0.2 M trehalose	0.4 M trehalose
1d	D-shaped	D-shaped	D-shaped
7d	Early-umbo	D-shaped	D-shaped
13d	Late-umbo	(*)	Early-umbo
17d	Pediveliger	(*)	Late-umbo
21d	Post-larva	(*)	(*)

3.5.5 Organogenesis

Characterisation of organogenesis during larval development was accomplished through detailed analyses of light microscopy photographs, which were taken for several representative larvae from replicate control and treatment tank. The observations revealed that 1dpf control D-stage larvae had indistinguishable features apart from the numerous cilia on the hinge end and digestive diverticulum was seen (Fig 3.11 - 1A). At 1 dpf, D-stage larvae in both 0.2 M and 0.4 M trehalose solutions respectively, displayed the same features as controls (Fig 3.11 - 2A, 3A). At 3 dpf, in the control larvae, style sac originated at the dorsal end of stomach near the hinge and velar retractor muscles started developing, digestive diverticulum became more distinguished (Fig 3.11 - 1B). However, larvae exposed to 0.2 M and 0.4 M trehalose showed abnormalities to various degrees in shell morphology and organogenesis, but if we

compare both the cryoprotectant solutions, then larvae exposed to 0.2 M trehalose show severe abnormalities, the digestive diverticulum had gained the shape of a nucleus and moved to one corner, which clearly indicates severe cryoinjury to the larvae (Fig 3.11 - 2B). In a separate picture of 3 dpf larva in 0.2 M trehalose, severe damage to digestive diverticulum was observed (Fig 3.12). Still another 3 dpf larva from 0.2 M trehalose show damage to digestive diverticulum as well as distorted shell shape, which is more elongated than normal D-shape (Fig 3.13). Larvae in 0.4 M also show abnormal organogenesis when compared with controls (Fig 3.11 - 3B).

As the organogenesis progressed further, at 7 dpf, control larvae changed their shape from typical D-shape to an early umbo stage. Organogenesis had progressed well with a well-developed mouth, esophagus and digestive diverticulum. These larvae had a small accumulation of microalgal content in the stomach, indicated the presence of brown pigmentation. Velum had developed well, with active cilia around it. Conversely, 7 dpf larvae exposed to 0.2 M trehalose showed distorted shells with undefined organs indicating the effect of cryoprotectant on its development (Fig 3.11 - 2C), and those exposed to 0.4 M trehalose appeared to have delayed organogenesis. Larvae still had D-shaped shell, with umbo just starting to develop (Fig 3.11 - 3C). Another 7 dpf larva exposed to 0.2 M trehalose show extensive internal damage as a result of cryoinjuries, though umbo had started to develop (Fig 3.14). At 13 dpf, control larvae had well-defined lobes within the digestive diverticulum in which great amount of microalgal accumulation was observed (Fig 3.11 - 1D).distinct velum retractor muscles were observed extending outwards from the umbo region to the velum, gill rudiment was seen developing on the ventral side. Few larvae exposed to 0.2 M trehalose progressed to 13 dpf, but due to low survivability and poor representation, no developmental observations were made. However, larvae exposed to 0.4 M trehalose did progress on to

early umbo stage (Fig 3.11 - 3D) exhibiting delayed organogenesis. Another 15 dpf larva from 0.4 M trehalose has successfully developed to late umbo stage but shell size is smaller with slow growth (Fig 3.15).

At 17 dpf, control larvae had developed to pediveligers with the presence of foot. Presence of minute cilia on foot was observed visually (Fig 3.11 - 1E), whereas the representative larvae exposed to 0.4 M trehalose were still at late umbo stage/ early pediveliger stage (Fig 3.11 - 3E). Both groups of larvae had large amount of microalgal content in the digestive diverticulum. Another day 17 larva from 0.4 M trehalose exhibit well developed velum and digestive diverticulum but still the growth was slower as compared to controls (Fig 3.16). At 21 dpf, control larvae had successfully metamorphosed and were characterised by the presence of an enlarged digestive diverticulum, gill rudiment, adductor muscle and a functional foot (Fig 3.11 - 1F). Later, the velum was totally reabsorbed as the larvae prepared to settle. Visual observations identified eye spots in most control larvae, but no eye spots were observed on larvae exposed to 0.4 M trehalose. The few specimens observed from this treatment still appeared to be at the late umbo stage.

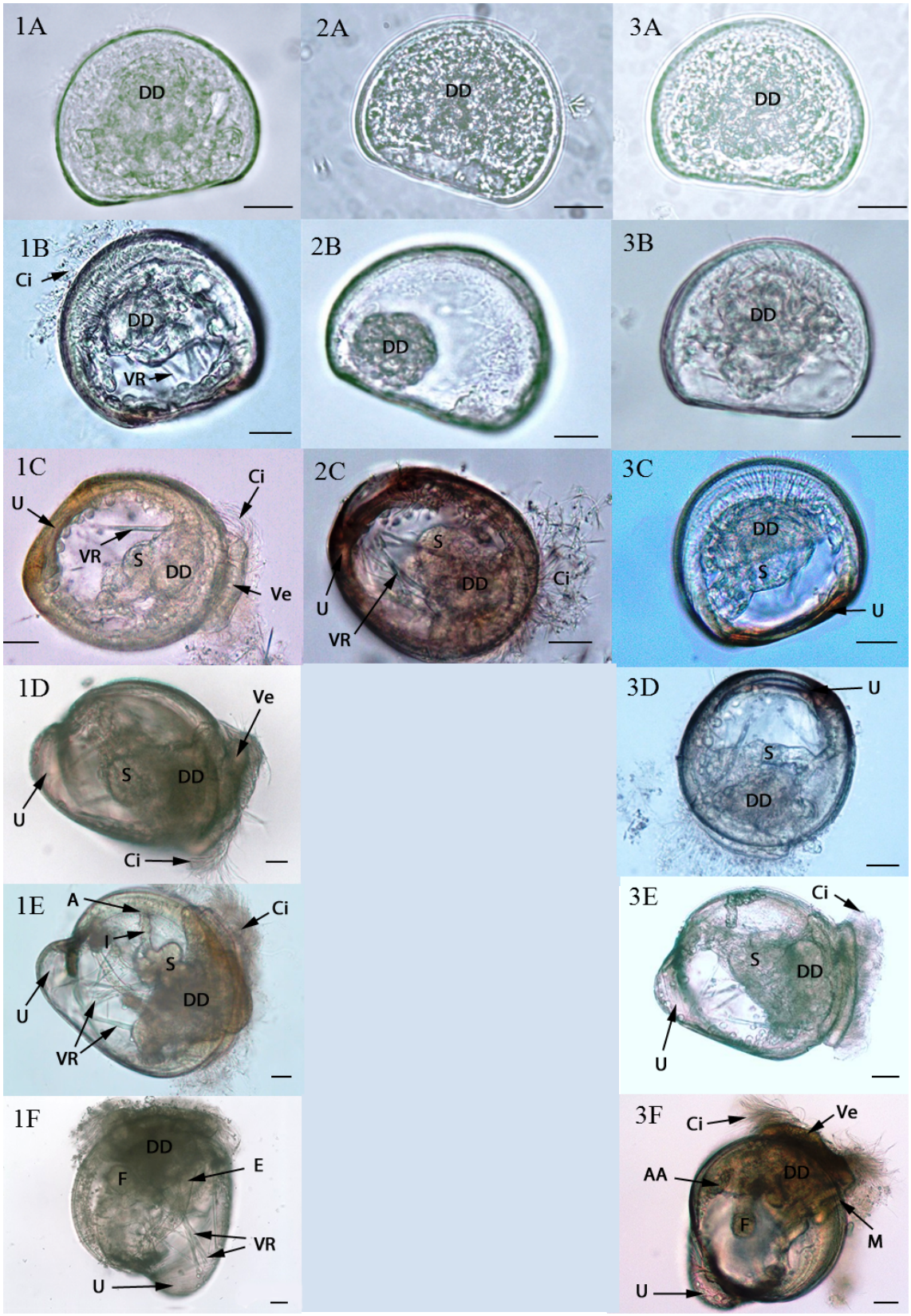

Figure 3.11.Light microscopy images of D-stage to late umbo stage *Crassostrea gigas* larvae showing overall organogenesis and microalgal content in stomachs over the rearing period within controls (1A- 1F) and larvae exposed to 0.2 M trehalose (2A- 2C) and 0.4 M trehalose (3A- 3F). A, B, C, D, E, F represent 1, 3, 5, 13, 17 and 21 days old larvae respectively. Abbreviations: A- Anus; AA- Anterior adductor muscle; Ci- Cilia; Ve- Velum; VR- Velar retractor muscles. Scale bars = 20µm.

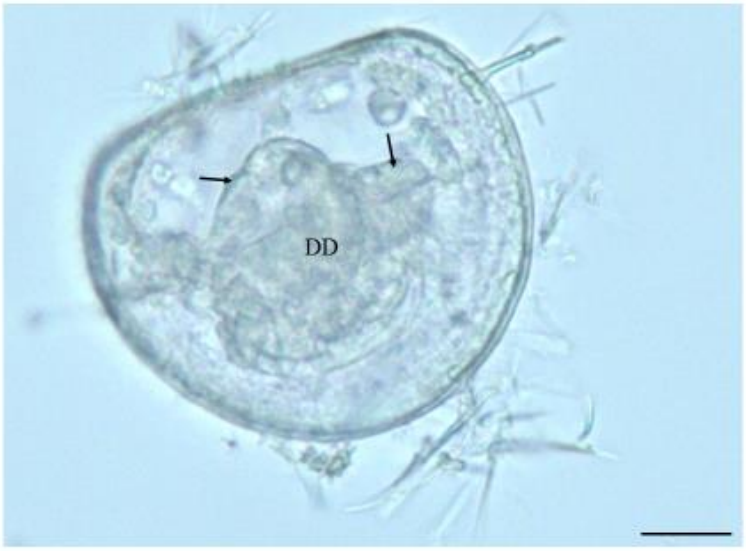

Figure 3.12.Light microscopy image of 3 dpf larvae in 0.2 M trehalose showing internal abnormalities. Abbreviations: DD- digestive diverticulum. Black unlabelled arrows show abnormalities. Scale bar = 20µm.

Figure 3.13.Light microscopy image of 3 dpf larvae in 0.2 M trehalose showing internal abnormalities. Abbreviations: DD- digestive diverticulum; Black unlabelled arrows show abnormalities. Scale bar = 20µm.

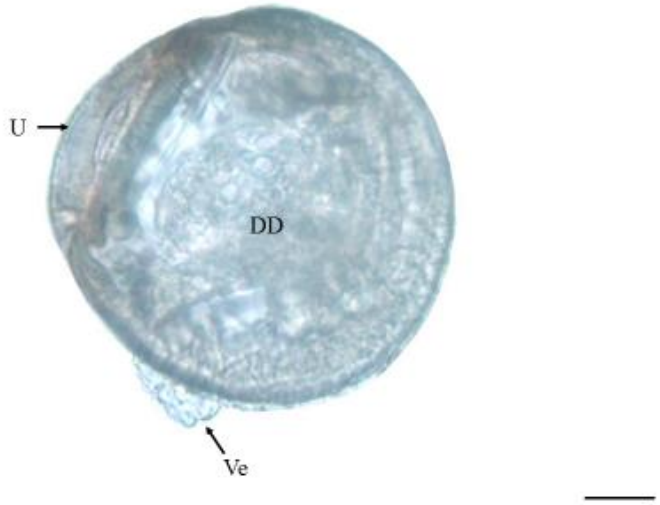

Figure 3.14.Light microscopy image of 7 dpf larvae in 0.2 M trehalose showing internal abnormalities. Abbreviations: DD- Digestive diverticulum, U- umbo, Ve- velum. Scale bar = 20µm.

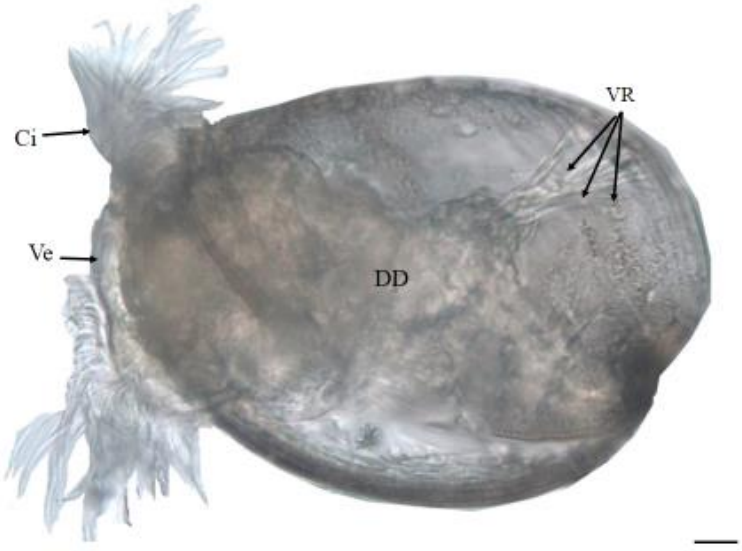

Figure 3.15.Light microscopy image of 15 dpf larvae in 0.4 M trehalose showing delayed organogenesis. Abbreviations: Ci- Cilia; DD- Digestive diverticulum; Ve- velum; VR- Velar retractor muscles. Scale bar = 20µm.

Figure 3.16.Light microscopy image of 17 dpf larvae in 0.4 M trehalose showing delayed organogenesis. Abbreviations: Ci- Cilia; DD- Digestive diverticulum; I- Intestine; U- Umbo. Scale bar= 20µm.

3.5.6 Neurogenesis

An effort to study neurology, using FMRFamide-like immunoreactive staining for neuropeptides in the larvae exposed to both 0.2 M and 0.4 M trehalose did not give satisfactory results as there was some misunderstanding in following the methodology, still the results found are compared below to the larvae from controls as explained in Chapter 2. Moreover, there were not many larvae left after performing light microscopy and scanning electron microscopy to make repeated observations.

At 1 dpf control larva had very little immunofluorescence. However, some activity was observed near mantle suggested to be mantle neuron. The apical neuron was not very clear. The peripheral nervous system had started to develop (Fig 3.17). However, 1 dpf larva exposed to 0.2 M trehalose showed negligible immunofluorescence activity around the margins, and there was no sign of apical neurons (Fig 3.20). Not enough larvae were available in 1 dpf eppendorf exposed to 0.4 M trehalose to make satisfactory observations.

At 7 dpf, the control larvae had successfully developed to the early umbo stage larvae with a brightly stained mantle nerve and velum showing rich immunoreactivity as the cilia are highly innervated by neurons (Fig 3.18). Mantle nerves completely merging into the mantle region were observed, but, in 7 dpf larvae exposed to 0.2 M trehalose the larvae were still in D-stage, and developed no further, as was seen in confocal image which show negligible FMRFamide like immunoreactive staining. (Fig 3.21). After 7 days, due to high mortality, very few larvae in the 0.2 M trehalose treatment survived and no observations were possible. However, in 7dpf larvae exposed to 0.4 M trehalose had much more FMRFamide-like immunoreactive staining compared to larvae exposed

to 0.2 M trehalose. These larvae has reached early umbo stage but were much smaller in size compared to those in controls. Mantle nerves were clear and high activity was observed around the velum suggesting high innervation of cilia with neurons (Fig 3.22).

As the larvae developed further, at day 21 dpf, the control larvae reached pediveliger stage nearing metamorphosis. A highly innervated foot was observed which indicates that the foot was rich in sensory neurons and play an important role in search for a suitable substratum for the larva to get attached and finally successful metamorphosis can happen. (Fig 3.19). A complete central nervous system along with peripheral nervous system should have been developed by now, but was not clear in the pictures, maybe due to poor absorption of the stain by the larva. In 21 dpf larva exposed to 0.4 M trehalose, the velum was reabsorbed and the larva was close to metamorphosis (Fig 3.23). The mantle nerve was observed with a few other sites of immunoreactivity, but again the results are not good enough to explain neurogenesis in detail.

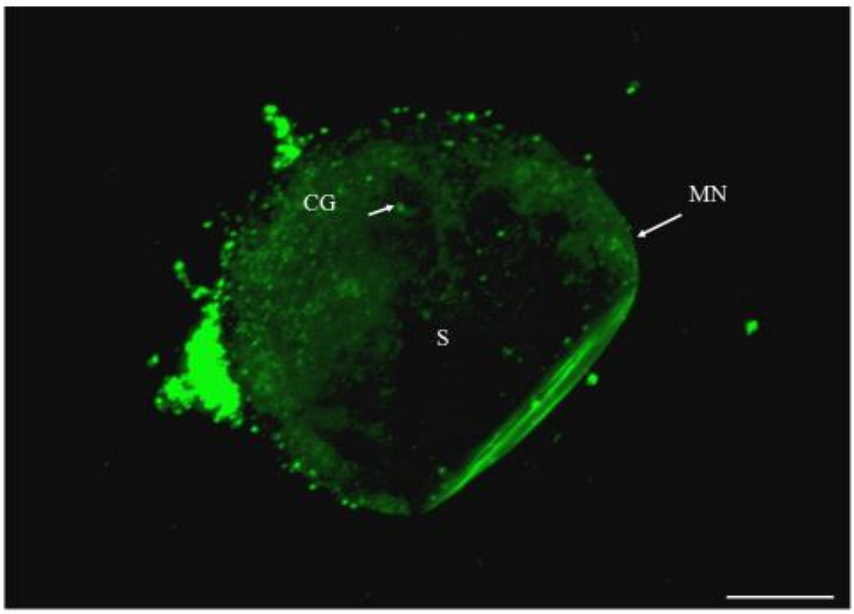

Figure 3.17.FMRFamide-like green immunoreactivity in a 1 dpf D-stage larva. Abbreviations: CG- cerebral ganglion; MN- mantle nerve; S- stomach. Scale bar = 20µm.

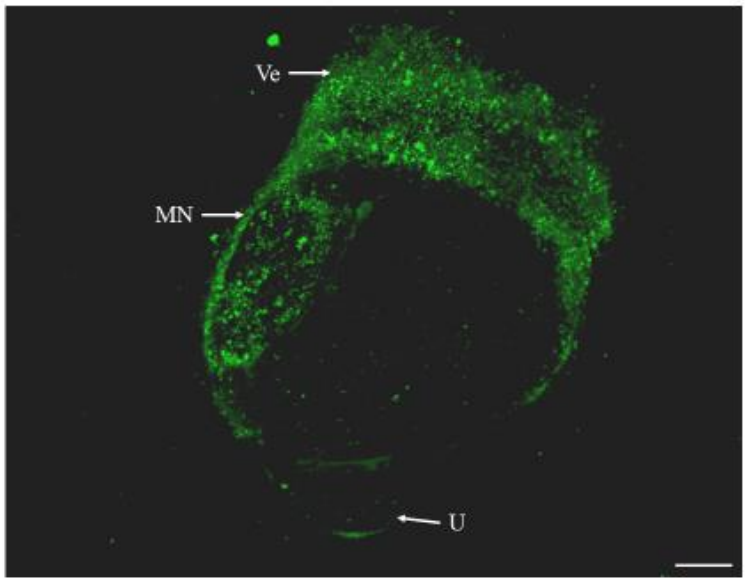

Figure 3.18.FMRFamide-like green immunoreactivity in a 7 dpf larva. Abbreviations: MN- mantle nerve; U- umbo; V-velum. Scale bar = 20µm.

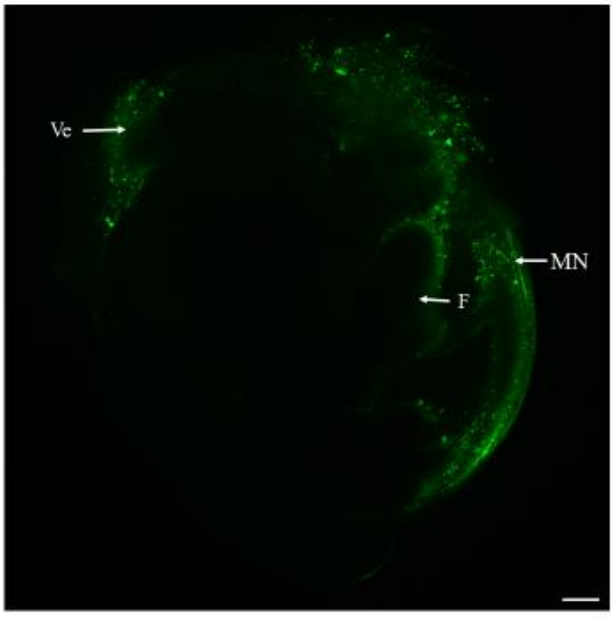

Figure 3.19.FMRFamide-like green immunoreactivity in a 21 dpf larva. Abbreviations: F- foot; MN- mantle nerve; V- velum. Scale bar = 20µm.

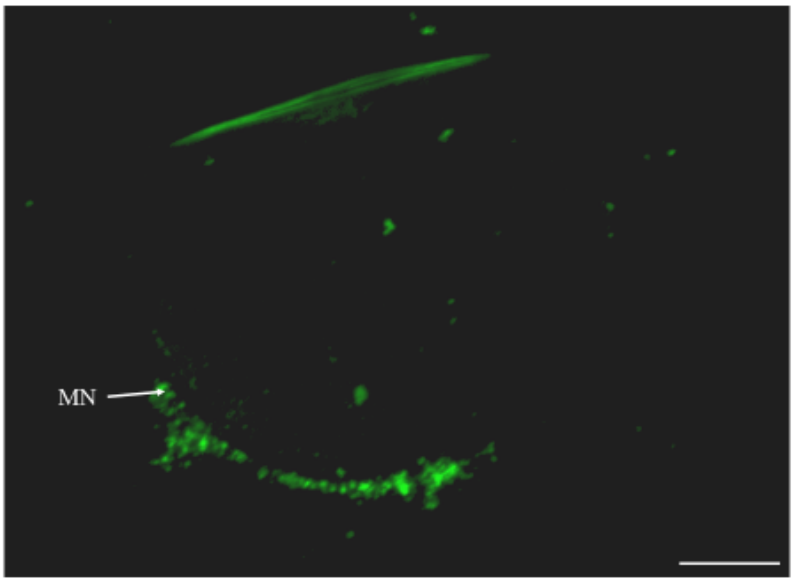

Figure 3.20.FMRFamide-like green immunoreactivity in a 1dpf D-stage larva exposed to 0.2 M trehalose. Abbreviations: MN- mantle nerve, Scale bar = 20µm.

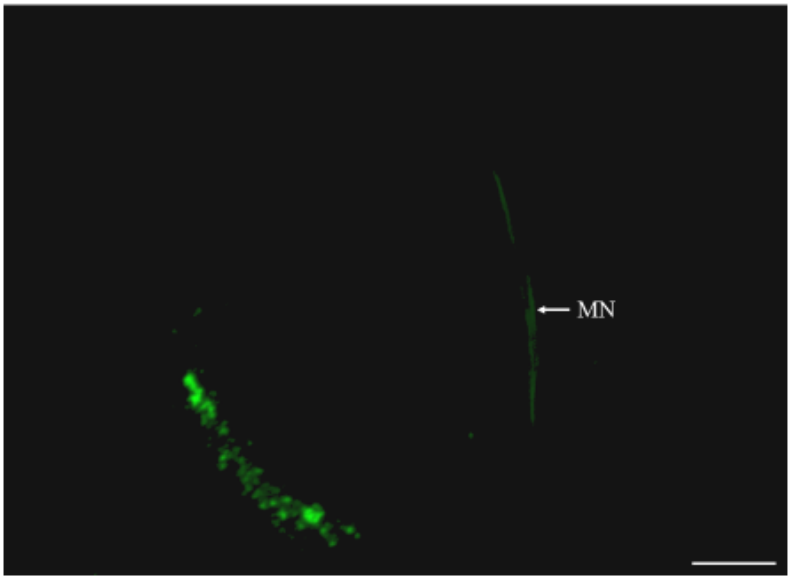

Figure 3.21.FMRFamide-like green immunoreactivity in 7 dpf larva exposed to 0.2 M trehalose. Abbreviations: MN- mantle nerve, Scale bar = 20µm.

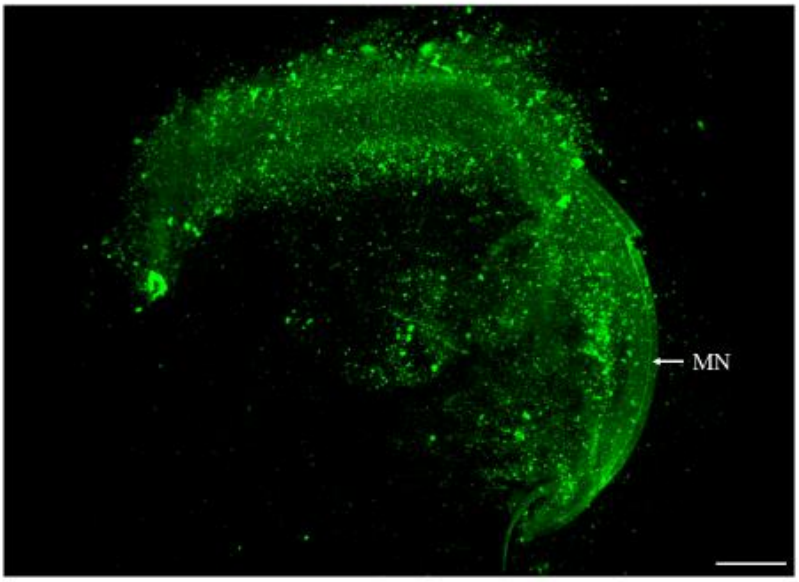

Figure 3.22.FMRFamide-like green immunoreactivity in a 7 dpf larva exposed to 0.4 M trehalose. Abbreviations: MN- mantle nerve, Scale bar = 20µm.

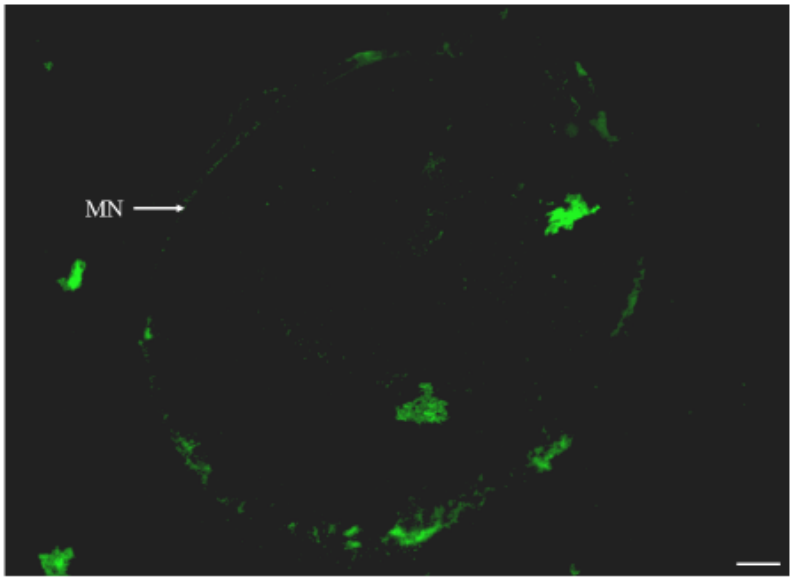

Figure 3.23.FMRFamide-like green immunoreactivity in a 21 dpf larva exposed to 0.4 M trehalose. Abbreviations: MN- mantle nerve, Scale bar = 20µm.

3.6 Discussion

Abundant literature supports a deep interest of researchers in cryopreservation of bivalve gametes, embryos and larvae. Cryopreservation of *Crassostrea gigas* sperm (Adams et al., 2004; Adams et al., 2011a; Dong et al., 2005; Smith et al., 2001), oocyte (Adams et al., 2011b; Salinas-Flores et al., 2008; Tervit et al., 2005) and embryos (Chao & Liao, 2001; Gwo, 1995; Lin et al., 1999; Renard, 1991) has been extensively investigated. Cryopreservation of *Crassostrea gigas* sperm was achieved decades ago through dedicated work of Lannan (1971), but its practical application was long due until Adams et al. (2004) developed a practical method for commercial spat production. Success is also been achieved in developing an optimum cryopreservation protocol for *Crassostrea gigas* oocytes (Tervit et al., 2005). Few studies show survival of Pacific oyster embryos after cryopreservation, but high mortality rates have been reported post-thawing (Gwo, 1995; Renard, 1991). To date, there are few studies reported on cryopreservation of Pacific oyster larvae at the D-stage larval stage, although later stages have been suggested to be more resistant to cryopreservation stress (Gwo, 1995; Nascimento et al., 2005; Suquet et al., 2012).

Cryopreservation is a valuable scientific tool which can be used in selective breeding in aquaculture as it enables the researchers to select the healthy stock which provides the genetic material to be stored and crossed at will. It provides the opportunity to the farmers to use the hatchery reared larvae as opposed to wild spat collection. As far as *Crassostrea gigas* is concerned, developing a successful protocol for cryopreservation of larvae, promises the year round availability of stock on demand reducing any natural or unnatural risk and ultimately improving the promising oyster productions in hatcheries. However, this task is not without its challenges as previous studies have

clearly highlighted the importance of optimised protocols for each species and cell type (Adams et al., 2011a; Adams et al., 2009; Bellas & Paredes, 2011; Paredes et al., 2012; Paredes & Bellas, 2009; Paredes et al., 2013; Smith et al., 2012; Wang et al., 2011). Moreover, the importance of evaluating cryopreservation success, not just immediately post-thawing, but over longer time frames has been recognised (Paredes et al., 2012; Wang et al., 2011). Based on previous studies, this study aims to investigate the effects of cryopreservation on subsequent larval development to post-settlement along with parametrical growth observations, such as shell length, survivability and feeding consumption. Overall, results indicate that there are significant differences observed in both the cryoprotectant treatments when compared with controls. Larvae exposed to 0.4 M trehalose performed far better than larvae exposed to 0.2 M trehalose, but both the larvae vary greatly from the controls, with controls developing exponentially better than the cryopreserved larvae in all the parametric tests as well.

3.6.1 Survivability

The survivability of cryopreserved larvae with the two different cryoprotectant solutions, 0.2 M and 0.4 M trehalose was significantly lower than that of the control larvae. This trend was maintained throughout the rearing period of 22 days. In general, a sharp decline was observed from day 1 to about 5 dpf, but these initial sharp declines are commonly encountered in hatchery rearing of larvae. Further moving to day 15, 100% mortality was observed in 0.2 M trehalose exposed larvae, whereas few larvae exposed to 0.4 M trehalose managed to grow until day 21 but the survivability was very low as compared to controls. Indeed, this trend was observed by Wang et al. (2011), who described a significant decline in survival of cryopreserved blue mussel (*Mytilus galloprovincialis*) between 3 dpf and 6 dpf. This low survivability trend was also

observed by Rusk et al. (2013), who described a significant decline in cryopreserved *Perna canaliculus* larvae, with only 0.03 ± 0.03% cryopreserved trochophores and only 0.5 ± 0.26% D-stage larvae successfully made it to 18 days but none of the cryopreserved larvae achieved settlement around 21 days. The CPA combination of 10% EG and 0.4 M trehalose was used in the study. Suquet et al. (2012) also reported high initial mortalities for *Crassostrea gigas* larvae that had been previously cryopreserved, and those larvae had lower overall survivability than comparative controls. The CPA used in the experiment was 10% EG with 1% PVP and 200 mM trehalose, prepared in Milli-Q water at the dilution rate of 1:1.

In the present study, unfortunately, the numbers of surviving larvae after 5 dpf in the treatments were too low to provide comparative data among treatments as all the larvae exposed to 0.2 M trehalose died by day 15. However, treatments with 0.4 M trehalose still had some viable larvae up to day 19 and 21. The most suitable treatment was found to be 0.4 M trehalose at 1˚C min^{-1}, which resulted in about 658 ± 570 surviving larvae on day 21, which were not very different from comparable control larvae. The optimum concentration of 0.4 M trehalose was also reported as beneficial when used in combination with 10% EG for *Perna canaliculus* trochophores (Paredes et al., 2012). The study also reported higher concentration of EG (15%) and a lower trehalose concentration was also found to be beneficial. Based on these studies, in our study we used the combination of 10% EG, 1% PVP and either 0.2 or 0.4 M trehalose to help reduce any cryoinjury. While working on Greenshell mussel oocytes, over the range of EG concentrations found that there was no benefit of increasing EG concentration and this CPA works best with combination of trehalose (Adams et al., 2009). The study revealed that the combination of 10. % EG along with 0.2 M – 0.4 M trehalose provides

the best protection against cryoinjury. This finding also supports the use of CPA combination in our study.

Many studies support the fact that cryopreservation protocols are species specific and the apparent toxicity of cryoprotectants is dependent on the type and concentration of cryoprotectant. Paniagua-Chavez and Tiersch (2001), while working on eastern oyster *Crassostrea virginica* sperm and larvae found that the best results were obtained using 10 % or 15 % propylene glycol. High rate of survivability was achieved using the CPA. The study claimed that the procedure developed were suitable for production of seed stock in commercial oyster hatcheries.

As far as cooling rates are concerned, again Paredes et al. (2012) used cooling rate of 0.5˚C min^{-1} and 1˚C min^{-1} and found that there was effect of cooling rates between 0.5 and 1˚C min^{-1}down to -35 ˚C on larvae developing to D-stage. These finding are similar to our findings as no significant difference in survivability was found among three different cooling rates. Our study shows the best cooling rate for *Crassostrea gigas* larvae developing to D-stage is 1˚C, however, optimising the cooling rate and CPA for D-stage larvae of *Crassostrea gigas* is yet to be determine through future experiments. This protocol was also supported by Paredes et al. (2013), while comparing cryopreservation protocol on *Crassostrea gigas* and *Mytilus galloprovincialis* larvae found that, the best results for oyster trochophores were obtained with survivability rate of 60.0 ± 6.7% , when the larvae were held at 0˚C for 5 min then cooled at 1˚Cmin^{-1} to 10˚C and held again for 5 min before cooling at 0.5˚C to -35˚C, held for 5 min and plunged into liquid nitrogen, using 10% EG. For mussels, again no significant differences were found when cooling at 0.5 ˚C min^{-1} or at 1 ˚C min^{-1} for CPA

combinations with 10% EG. Survivability percentage of 48.9 ± 7.6% was observed in mussels.

Another aspect of determining the quality of post thaw larvae used by Suquet et al. (2012) was calculating the Average Path Velocity of D-stage larvae using CASA-Computer Assisted Sperm Analysis System. The study revealed a reduced Average Path Velocity of thawed cryopreserved *Crassostrea gigas* larvae as compared to control larvae. Suquet et al suggested that the larval movement velocity can be used as an assessing tool for the quality of cryopreserved larvae. However, this swimming behaviour was observed only visually in the current study as no behavioural tests were performed. We suggest that analysis of Average Path Velocity of D-stage larvae could be used as quality assess tool for *Crassostrea gigas* larvae in cryopreservation experiment in future along with other parametric tests. We also emphasise the determination of best cryopreservation combination that yields best survival rate with good quality post-thaw larvae in future experiments.

3.6.2 Shell Length

Shell length in all the treatments as well as control show general increase in length over the 22 days rearing period with wide variations. The growth pattern observed revealed, these variations were irrespective of the cooling rate conditions. Control larvae were bigger and possess normal shape and developmental features whereas larvae exposed to 0.4 M trehalose were quiet smaller in length and were slightly oval in appearance. Conversely, larvae exposed to 0.2 M trehalose exhibited numerous abnormalities and very slow shell growth resulting in considerably small shell length. The results found in the present study suggests that 0.4 M trehalose cryoprotectant solution was most

appropriate but still not sufficient to protect the larvae from cryoinjury. This shell length variations is well understandable as the effect of cryoprotectant and has been reported by many researchers. While doing the comparative study in oysters and mussels, Paredes et al. (2013) found that the size of D-stage *Crassostrea gigas* larvae obtained from thawed trochophores were 10.3% smaller than the unfrozen control larvae as compared to *Mytilus galloprovincialis* larvae which were 17.9% smaller than unfrozen controls. Another study conducted on *Perna canaliculus* larvae revealed significant differences in shell length between cryopreserved trochophore larvae and controls. The controls were almost 10% bigger than the cryopreserved larvae (Paredes et al., 2012) and only 2.8% of frozen trochophores were able to develop into competent veligers.

In a separate study on *Perna canaliculus*, Rusk et al. (2013) found the shell length of cryopreserved trochophores and D-stage larvae were smaller as compared to the controls. These findings were similar to our present study, however, in another study (Wang et al., 2011) reported the shell length difference in frozen D-stage larvae and the controls of the blue mussel *Mytilus galloprovincialis* to be insignificant, as no major differences were observed except on day 6. These findings were contrary to our findings as in our present study, we observed the shell length difference from 1dpf throughout the total 22 day larval rearing period. However, not much data is available in literature regarding shell length variations due to cryopreservation as most of the work is concentrated to survival percentage only.

We therefore encourage more research needed to assess the effects of cryoinjuries on shell length of *Crassostrea gigas* larvae as smaller shell length could be one of the reasons for delayed organogenesis. From the prior studies, we can conclude that cryoinjuries to the internal organs like shell gland are accountable for smaller shell

length, as shell gland and mantle, both are responsible for secretion of shell layers namely PI and PII respectively. So any injury to these organs effect the shell length directly and indirectly to the delayed organogenesis, hence, metamorphosis.

3.6.3 Feeding consumption

During the first 7 dpf, feeding consumption patterns for controls and the treatments were almost same, however between 7 dpf and 19 dpf, a significant variation was observed with higher consumption for control larvae. At 5 dpf, a large peak in consumption was observed in controls and all the treatments which corresponds to high mortality period. Previous studies have revealed the similar trends and accounted the bacterial loads associated with live feeds to high larval mortalities, especially during early larval stages (Gomez-Leon et al., 2008; Rico-Villa et al., 2009; Torkildsen et al., 2005). The feeding consumption peak observed at 5 dpf was also observed by Rusk et al. (2013) in *Perna canaliculus* larvae during the cryopreservation experiment.

In the present study, controls larvae were able to maintain higher survival rates and feeding consumption rate compared to the treatments throughout the experiment, indicating their superior performance as compared to treatment larvae. Interestingly, drops in feeding consumption level on days 9 and 11 are unexplained, and may be attributed to low survival rate and high variation in population behaviour. It was observed that in 0.2 M trehalose treatment feeding consumption and survival rate was the lowest indicating maximum cryoinjuries in this treatment. But in controls and 0.4 M trehalose treatment with 1˚C min^{-1} cooling rate, a consistent feeding consumption level had been achieved. These results were also supported by observations of full gut contents in the representative larvae, especially for controls and 0.4 M trehalose, 1˚Cmin^{-1} treatment. Low feeding consumption rate or low microalgal content in the larval guts of all the other treatment larvae in the later stages indicates the poor larval performance and onset of mortality (Rusk et al., 2013; Wang et al., 2011). These

findings were supported by our studies as the treatments with low feeding consumption level were found to have lower survivability rates and high mortality in the later stages.

Indeed, (Rusk et al., 2013) suggested that lack of or low feeding activity may take place as a result of cryoinjuries to important feeding organs such as the velum, digestive diverticulum and stomach. The above observation was also done in the present study and we found out that all the 0.2 M treatments and some of the 0.4 M trehalose treatments had serious damages to the feeding organs, while controls and 0.4 M trehalose, 1˚C min^{-1} show no damage to feeding organs. This observation supports that the 0.4 M trehalose, 1˚C min^{-1} was the best cryopreservation for Pacific oyster larvae.

3.6.4 Shell morphology

Shell morphology of the cryopreserved larvae in both the trehalose treatments in our study was different to the control larvae. Control larvae were bigger and symmetrical in appearance, with appropriate shell length. Larvae at 1 dpf show normal PI region and as the larval development progressed further, PII region was secreted by shell gland and the mantle representing normal growth. Postero dorsal notch was observed in all the control larvae. Whereas in larvae exposed to 0.4 M trehalose exhibit smaller shell length and were slightly oval in appearance. Though, postero dorsal notch was observed in most of the larvae. However, if we compare controls with 0.2 M trehalose exposed larvae, these cryopreserved larvae show serious abnormalities to the shell surface. A few larvae had wrinkled appearance on the outer shell surface on the punctuate region of the PI layer extending vertically towards the mantle edge. The shape of some larvae were even distorted. The shell was not properly formed in some samples. All these larvae were destined to die in near future. Very few larvae further developed PII layer,

that too very late in the development. However, no larvae managed to reach day 15. These observations are similar to that reported for *Perna canaliculus* larvae that had been cryopreserved in the similar manner (Rusk, 2012; Rusk et al., 2013). In the study, wrinkle type appearance was observed on the surface of the shell, also some unsymmetrical left and right valves were observed. The study suggested the abnormalities to the shell may be due to cryoinjuries to the shell gland and mantle, which are responsible for the secretion of PI and PII layers of the shell. While studying the effect of developmental stage, seawater concentration and rearing temperature on the cryopreserved larvae of *Crassostrea gigas*, rough wrinkled surface was reported on the shell surface in post-thaw larvae which was considered malignant and responsible for the death of larvae (Usuki et al., 2002).

Limited amount of literature describing abnormalities of bivalve larval shell morphology emphasise a lot to be done in this field. In a study performed on Sydney rock oyster *Saccostrea glomerulata*, scanning electron microscopy images revealed abnormalities to the shell surface due to low pH of water (Watson et al., 2009). In the study, controls show normal shell development with smooth and rounded shell with even growth. However, at lower pH the larval shell deposition was not smooth and show wrinkled appearance, moreover shells show deformity on the edges, indicating potential problem with shell deposition or periostracum formation. Though the conditions in our experiment are very much different to the above study but shell abnormalities are almost the same. In another study performed by Nascimento et al. (2005) on *Crassostrea rhizophorae,* described similar shell abnormalities. The shells were irregular or distorted in shape and sometimes not even completely developed. Findings in our study provides a platform to other researchers to study the effect of cryopreservation on shell morphology of various bivalve larvae and we emphasise that

further research is much needed to determine the exact cause of shell morphology and different types of shell deformities.

3.6.5 Organogenesis

Organogenesis show clear differences in control and all the treatments, 0.2 M trehalose and 0.4 M trehalose exposures, regardless of the cooling rate. Analyses of light microscopy indicates serious cryoinjuries to 0.2 M trehalose exposed larvae with high mortality rate, while larvae exposed to 0.4 M trehalose show delayed organogenesis, oval shell appearance and shorter shell lengths, but larvae were very much similar to control ones. Detail study of light microscopy pictures revealed that controls had normal development and the larvae were able to progress to the settlement stage where eye spots, gill rudiment and functional foot were observed. Whereas larvae in treatments show slow growth and cryoinjuries were evident especially in 0.2 M trehalose exposed larvae, which show developmental problems with certain organs, such as velum and digestive diverticulum. Similar larval abnormalities have been reported for cryopreserved mussel (*Perna canaliculus*) larvae (Rusk et al., 2013) and Sydney rock oyster (*Saccostrea glomerulata*) larvae (Liu & Li, 2008).

Rusk et al (2013), while studying the effect of cryopreservation on *Perna canaliculus* observed delayed organogenesis accompanied by some minor deformities. In the study no cryopreserved larvae was able to reach settlement. Liu and Li (2008) reported cryoinjuries to certain organs resulting in delayed development of Sydney rock oyster. The study held these cryoinjuries responsible for death of the larvae. This observation is supported by results in our present study, where we find 100% mortality in 0.2 M trehalose exposed larvae with acute damage to certain organs like velum and digestive

diverticulum. In the present study, we observed delayed organogenesis in 0.4 M trehalose exposed larvae, as, at 7 dpf, they were still at D-stage, while controls had progressed to early umbo stage. Furthermore, at 17 dpf when controls reached pediveliger stage 0.4 M trehalose treated larvae were managed to reach late umbo stage. Concept of delayed organogenesis was also explained by Wang et al. (2011), who observed less than 10% cryopreserved *Mytilus galloprovincialis* developed an eye spot at 16 dpf, however, at 21 dpf, almost 78% of cryopreserved larvae had developed eye spot, this observation suggests the delayed organogenesis in the cryopreserved larvae. In case of mussel (*Mytius edulis*) and scallop (*Pecten maximus*), low temperatures also delayed organogenesis as was reported by Beaumont and Budd (1982).

Usuki et al. (2002), observed disruption in the plasma membrane, thought to be caused by intracellular ice formation (IIF), which was responsible for the damage to microvilli and hence less larval motility. Overall, in our study, 0.4 M trehalose treatment at the cooling rate of 1˚C min^{-1} seems appropriate for minimising the cryoinjuries and post-thaw damage, but further research is required to optimise it and more detailed investigations are needed to study the damage at cellular level as our study was limited only to the organ level.

3.6.6 Neurogenesis

Due to lack of study material and misunderstanding of methodology, the results for the effect of cryopreservation in neurogenesis in this study were insufficient to make definitive observations. However, few images that are available indicates that larvae in the controls outperformed both the treatments, starting from D-stage through to the settlement stage. However, 0.4 M treatment larvae also show more or less satisfactory

development but 0.2 M larvae had performed exceptionally low. Our observations go in accordance with neurogenesis studies performed on *Perna canaliculus* in which the cryopreserved larvae show very little FMRF amide like neuroactivity as compared to controls (Rusk, 2012). The study also reported delay in neurogenesis in cryopreserved larvae and the reason for delay was attributed to delay in organogenesis. Our study was the first in its own to attempt the study of cryopreservation on the neuronal process of *Crassostrea gigas* during the larval development but, unfortunately lack of good results discourage to go in detail discussion. However, it is suggested a detail investigation of neurogenesis in cryopreserved larvae of *Crassostrea gigas* will help understanding vital processes involved in the larval development such as settlement cues and metamorphosis.

3.6.7 Summary

In summary, there were significant differences observed between controls and all the treatments, with respect to all parametric data, including shell morphology as well as in organogenesis. Neurogenic effect were not determined based on the limited data. The differences in control and treatments are most likely due to cryoinjuries sustained during the chilling and thawing process. Cryopreservation of *Crassostrea gigas* larvae has been attempted a few times so far, and larval survivability is considered as a major obstruction. In this study, larval survivability immediately after post-thaw was satisfactory, but as the larvae underwent further development, the cryopreserved larvae show significant difference in survivability, shell length and feeding consumption from the controls.

Further investigations in our study revealed abnormalities in shell development and organogenesis. Larvae exposed to 0.2 M trehalose performed exceptionally low, irrespective to the cooling rates, and were not able to develop properly. Even after 5 days of development, when control larvae successfully made it to early-umbo stage, 0.2 M exposed larvae show no development and still exhibit D-stage, furthermore, these larvae underwent 100% mortality before day 15. This suggests maximum cryoinjuries in 0.2 M treatment. The larvae exposed to 0.4 M trehalose, especially at the cooling rate of 1˚C min^{-1} performed well, but still differed from those in controls, specifically in shell size, appearance and exhibited delayed organogenesis. This indicates the potential of cryopreserving *Crassostrea gigas* larvae in future using 0.4 M trehalose as cryoprotectant solution, if the survival rates can be improved. In addition, optimising the cooling rate for higher long term survival is required. While the larval survivability was low in both the treatments with-in our study, the detailed observations of external and internal larval development were crucial to identify the quality and viability of these larvae.

To identify cryoinjuries, not just after post-thaw but later in the development, shell morphology, organogenesis and neurogenesis observations are powerful tools. Thus, the findings in this research provide an excellent starting point for further optimising cryopreservation protocols for D-stage Pacific oyster (*Crassostrea gigas*) larvae.

CHAPTER 4 General Discussion

4.1 Discussion

The research work on *Crassostrea gigas* larvae so far is confined to its population dynamics (Diederich et al., 2005; Wang et al., 2007), larval shell morphology (Christo et al., 2010; Dinamani, 1976), and settlement behaviour (Fitt et al., 1990; Yamaguchi, 1994). As far as cryopreservation is concerned, extensive investigations have been conducted to cryopreserve the gametes of this species and fortunately researchers have successfully achieved this aim (Adams et al., 2004; Tervit et al., 2005). Efforts to cryopreserve the embryos of this species have yield mixed results. According to previous findings, D-stage larvae show more resilience to cryoinjuries than earlier embryo stages (Adam's personal observations). Furthermore, recognition of the importance of evaluating cryopreservation success over a longer frame of time, and not just immediately post-thawing inspired the undertaking of this study. Moreover, there is little knowledge available regarding feeding behaviour, growth of shell with respect to larval age and most importantly organogenesis for this species. Limited information exists about how the shell grows and how the organs are formed inside the shell under a normal environment, and we presently cannot estimate the effect of challenges faced by this species due to change in environment and unnatural stress conditions (introduction of cryopreservants). In New Zealand, this oyster holds a special place in aquaculture industry because of its high export prices and high nutritional value. Apart from this, Pacific oysters help to maintain the quality of water in our estuaries because of its filter feeding behaviour. Last, but not the least, this oyster has a cultural significance because it is a source of traditional (Kai-moana) harvesting.

This thesis was developed from a research effort to investigate the shell morphology, organogenesis and neurogenesis under normal hatchery conditions during the total rearing period of 22 days, then supplementing the research by studying the effect of introduction of cryopresevative agents on the different aspects of larval development. Thus, findings of this thesis, describe the detailed larval development and the effects of cryopreservation on post-thaw larvae over the total rearing period of 22 days under hatchery conditions for *Crassostrea gigas* using multi-disciplinary approach. This research work enhances our knowledge and fills the gap which was present to date regarding the detail larval development of this species. The findings from this work increases our knowledge about the invertebrate larval biology and cryobiology, not only for this species but other relevant species of bivalve molluscs.

During the present study, various aspects of shell morphology were investigated, such as shell length, formation of PI and PII layers from D-stage larvae to post settlement under normal hatchery conditions. The findings from this study can be used to condition the broodstock as well as to characterize the developmental stage of wild stock. To study the aspect of shell morphology, scanning electron microscopy was used as it overcomes the limitations of light microscopy. Analyses of light microscopy pictures helped to catalogue various stages of organogenesis in fine details because of transparent shells of *Crassostrea gigas* larvae. Our study identified specific characteristics of larvae during the developmental process which were not mentioned in any previous work thus filling the gap in our first step of study. The findings have increased our knowledge about the biology of *Crassostrea gigas* larvae manifolds, as limited literature is available regarding the above facts.

The second step in the research was to study the effect of cryopreservation on shell morphology, which demonstrated various abnormalities in the shell structure, shape, secretion of PI and PII, such as wrinkled appearance on the shell surface as well as failure of 0.2 M trehalose cryopreserved larvae to secrete PII layer. These findings provide a foundation for upcoming work based on cryopreservation of *Crassostrea gigas* larvae. This study also catalogues various organogenesis abnormalities during larval development such as delay in organogenesis, less developed velum, damage to digestive diverticulum, which has never been documented before in case of *Crassostrea gigas* larvae. Findings of this study can be used by other researchers, while optimising the cryopreservation protocol for *Crassostrea gigas* larvae as well as for other related bivalve molluscs larvae. Thus, in addition of providing a catalogue of normal developmental stages including survivability, feeding consumption, shell length, organogenesis and the study also provides a comparison of developmental processes between normal and cryopreserved larvae under influence of cryopreservation solution. In addition to this as our study included 2 different cryopreservation solutions 0.2 M and 0.4 M trehalose respectively, providing results, which vary considerably among themselves indicating, the effect of cryoprotectant solution depends a lot on its concentration used, though all the other factors remain the same. The study also included an aim to investigate neurogenesis during the larval development from D-stage through to settlement period, then comparing them to cryopreserved larvae, but due to lack of enough study material, and poor staining, the results found were not satisfactory enough. Hence, the study emphasises the incorporation of neurogenesis in further experiments. The results and the abnormalities described in our present study can be referred by future cryobiologists to study effects of cryoinjuries on Pacific oyster and other related bivalves.

Cryopreservation of *Crassostrea gigas* larvae includes a sequence of steps, and any deficits, how minor it is in any step can lead to loss of viable larvae, as was observed in the present study. Larvae exposed to 0.4 M trehalose performed really well as compared to 0.2 M trehalose exposed larvae, but when it came to three different cooling rates larvae exposed to 0.4 M trehalose at the cooling rate of $1^{\circ}Cmin^{-1}$ performed well as compared to the other two cooling rates of 0.5 and $2^{\circ}Cmin^{-1}$ respectively. Hence, findings in this thesis demonstrates the importance of optimum cooling rate and other procedures during cryopreservation. However, our study indicates that the use of 0.4 M trehalose as a cryoprotectant solution along with ethylene glycol and PVP may have good application in hatchery production, but further optimisation of the protocol is must in order to increase the percentage survivability of viable larvae.

As far as New Zealand aquaculture industry is concerned, success in cryopreserving viable *Crassostrea gigas* larvae would be of great value. As Pacific oyster along with King salmon and greenshell mussel, is the backbone of aquaculture sector but presently, many farmers depend on wild-caught spat (Juveniles) to on-grow. As we know recent outbreaks of the Ostreid herpes virus (OsHV-1) have caused mass juvenile mortality in New Zealand, affecting juvenile oysters (Renault et al., 2012). Moreover, wild spat is always under the risk of predation and other natural disasters (Young, 2009). Under these circumstances, cryopreserved larvae can be alternate source of supply to the farmers all the year around. Cryopreservation techniques also provide scientists the ability to develop healthy family lines for interbreeding resulting in more viable larvae. Another important aspect of cryopreserved larvae of *Crassostrea gigas* is its use as a standard to determine the effect of future habitat changes on the species. The cryopreserved veliger larvae of Pacific oyster along with the Manila clam has been used to study the effect of contaminants along a well-defined Northern sea pollution gradient

(McFadzen, 1992). Cryopreservation of gametes, embryos and larvae of various species are highly recommended as these cryopreserved species may be used as standard experimental organisms in future studies (Chao & Liao, 2001).

Considering another point of view, in research projects which are collaborated with commercial hatcheries (such as the present study), where fertilisation trials are limited due to space and money constraints and primary focus is on production, we suggest that evaluation of lipid content of eggs during spawning should be taken into consideration before fertilisation followed by cryopreservation protocol as lipid content is a very good indicator of the health of egg as suggested by Soudant et al. (1999) and Gallager et al. (1986). Analyses of swimming behaviour of larvae is another aspect worth exploring and supplementing the findings with cryopreservation experiments can provide interesting results. Swimming behaviour may prove as an important tool as a general indicator of cell/tissue damage due to treatment effects as was suggested by Suquet et al. (2012). A reduced average path velocity in post-thaw larvae of *Crassostrea gigas* indicated poor quality of post-thaw larvae. This aspect should be included while assessing the effect of cryopreservation on any related invertebrate larvae, as this indicates the quality of cryopreserved larvae at D-stage only indicating the researchers to further optimise their protocol at very early stage.

This thesis also calls attention on the importance of cumulative effects on the larval development studies of any species undergoing the challenge of cryopreservation process, not just post-thaw but over a longer period of time as few side effects comes to the surface later in life, which are not visible or quantitative immediately after post-thaw. Also, we emphasise on the detailed study of neurogenesis in *Crassostrea gigas* larvae, as the effort to study this aspect in the present study could not yield satisfactory

results. This new technique could be very fruitful in studying the various mechanisms in larval behaviour as well as in studying the settlement cues, which is attracting a lot of researchers these days.

In summary, this thesis shows the potential of cryopreserved Pacific oyster larvae for future commercialization. This project also provides a framework for the development of cryopreservation protocol in other aquatic species which are of some importance in aquaculture sector. The findings in present study enhances our knowledge in understanding the Pacific oyster's larval developmental biology and the effect of cryopreservation on the larval development which completes the ultimate goal of this research work. Major issues raised by our study, for example need to optimise cooling rate will help the future cryobiologists to overcome the issues. Further investigations in this field as advised will prove highly beneficial to the aquaculture sector from commercial point of view worldwide.

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